ENZYMATIC AND ELECTROPHORETIC METHODS FOR CARBOHYDRATE DETERMINATION USING MICROSYSTEMS

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ABBREVIATIONS

µTAS	Micro total analysis
A	Ampere
AA	Ascorbic acid
AMPS	2-Acrylamido-2-methyl-1-propanesulfonic acid
APS	Ammonium persulfate
APTES	3-Aminopropyltriethoxysilane
Aux	Auxiliary or counter electrode
BIS	N, N`-methylenebis(acrylamide)
BR	Buffer reservoir
BSA	Bovine serum albumin
BW	Buffer waste
C&L	Clark & Lubs
CE	Capillary electrophoresis
CEC	Capillary electrochromatography
CIEF	Capillary isoelectric focusing
CITP	Capillary isotachophoresis
CTAB	Cetyltrimethylammonium bromide
CGE	Capillary gel electrophoresis
CMC	Critical micelle concentration
CVC	Critical vesicle concentration
CZE	Capillary zone electrophoresis
DDAB	Didodecyldimethylammonium bromide
FAD	Flavin adenine dinucleotide
FIA	Flow injection analysis
FI	Floating
g	Gram
GA	Glutaraldehyde
Gnd	Ground
GOD	Glucose oxidase
h	Hour
HCl	Hydrochloric acid
H_2O_2	Hydrogen peroxide
HV	High voltage

I	Current
i.d.	Inner diameter
K	Kilo
L	Litre
m	mille
M	Molar
MAPTS	8-Methylacryloxypropyltrimethyl silane
MEKC	Micellar electrokinetic chromatography
MES	2-[N-Morpholino]ethanesulfonic acid
min	Minute
MW	Molecular weight
NADH	Nicotinamide adenine dinucleotide
NaOH	Sodium hydroxide
^{o}C	Celsius
PAA	Polyacrylamide
pI	Isoelectric point
PMMA	Poly(methylmethacrylate)
Pt	Platinum
RE	Reference electrode
s	Seconds
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SR	Sample reservoir
SW	Sample waste
Temed	N,N,N`,N`-Tetramethylethylenediamine
Tris	Tris(hydroxy methyl)-aminomethane
UV	Ultraviolet
V	Volt
VSA	Vinylsulfonic acid
WE	Working electrode
YSI	Yellow Springs Instrument

1 TOPIC OF THE THESIS

Carbohydrates are the major energy delivering compounds for all living organisms without photosynthetic activities. The most important among them is D-glucose, as it is not only an important energy source but also a possible precursor for the biosynthesis of amino acids, nucleotides, fatty acids or cofactors required for growth of the organism. That is why a sufficient supply of cells with glucose or other carbohydrates, which can be feed into the glucose metabolic pathways, is essential for growth and survival of cells. In humans the glucose concentration in blood is strongly regulated by regulation of reactions leading to glucose consumption, glucose storage (as glycogen) or degradation of storage products.

Malfunction of these regulating cascades leads to illness, such as diabetes mellitus. Other metabolic diseases leading to an intolerance for certain carbohydrates are related to not properly regulated activities of enzymes involved in carbohydrate metabolism. The major source of carbohydrates for humans is food. That is why carbohydrate analysis, and in particular glucose analysis, is of importance in medical diagnosis and food analysis, and a number of suitable analytical procedures were developed ranging from chemical reactions to chromatographic or electrophoretic separations and enzymatic assays or enzyme electrodes. For routine analysis methods should be simple and fast deliver quantitative data and require minimal amounts of sample. That is why the combination of enzyme reactions with miniaturisation technologies is of increasing importance. Miniaturisation is achieved either by the production of miniaturised enzyme electrodes using for example screen-printing technology for electrode production or by the so-called lab-on-a-chip-conception, which analytical assays are performed in a capillary network of polymer chips.

A major issue of all analytical procedures is the elimination of matrix effects originating from the complex composition of samples, such as food, blood or serum.

Thus, the aim of the present work was the improvement of previously developed screen-printed electrodes with respect to elimination of matrix effects and the development of glucose assays in labs-on-chips, i.e. in microchips comprising a simple capillary system. The investigations should allow the evaluation of the possibilities of both approaches with respect to carbohydrate, in particular glucose, determination in complex samples.

2 STATE-OF-THE-ART

2.1 Carbohydrates

Carbohydrates are the main energy source for the human body. Chemically, carbohydrates are organic molecules from carbon, hydrogen and oxygen with a general formula $C_n(H_2O)_n$ (also called hydrates of carbon). Animals (including humans) break down carbohydrates during the process of metabolism to release energy. As an example, the complete chemical conversion/oxidation of glucose is shown:

$$6O_2 + C_6H_{12}O_6 \rightarrow 6CO_2 + 6H_2O + 2840 \text{ kJ/mol} \quad (2.1)$$

Carbohydrates are produced by plants using photosynthesis as energy delivering process. Plants harvest energy from sunlight to run the reaction described above in reverse.

Carbohydrates can be divided into monosaccharides, oligosaccharides, and polysaccharides depending on the number of sugar units that are linked together. Monosaccharides cannot be hydrolysed to smaller units. Two of the most common monosaccharides are glucose and fructose. Glucose is the primary form of sugar stored in the human body for energy. Fructose is the main sugar found in fruits. Both glucose and fructose have the same chemical formula ($C_6H_{12}O_6$) however they have different structures as seen in Fig 1.1.

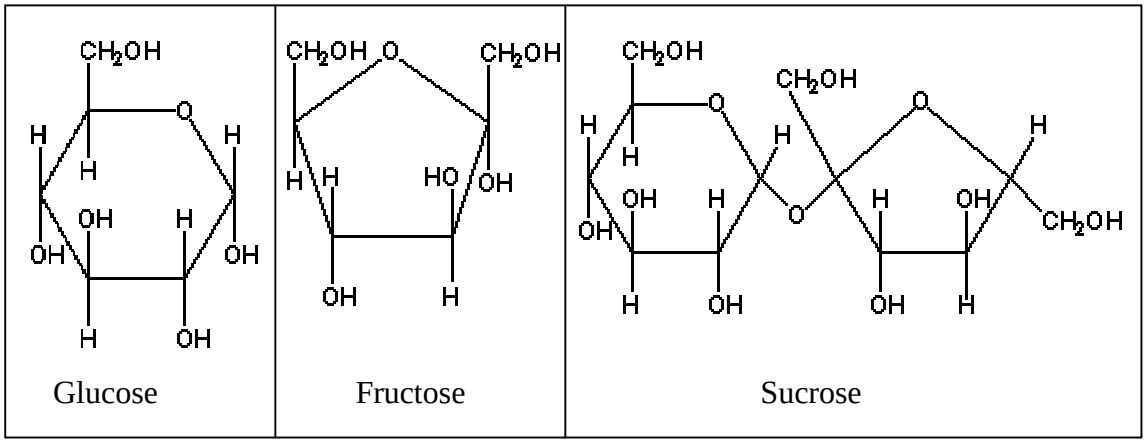


Fig. 1.1 Chemical structure for some carbohydrates.

Oligosaccharides contain 2-10 monosaccharide residues joined by glycosidic linkages, of which disaccharides are the most important. Disaccharides contain two sugar units, for example; the common table sugar is sucrose containing a glucose and a fructose unit (Fig 1.1). Polysaccharides are polymers of high molecular weight consisting of more than 10 monosaccharide units with the majority of the naturally occurring polysaccharides containing several thousand units. They are used for the storage of excessive glucose, such as starch in

plants or glycogen in animals, or as structural elements, which cannot be metabolised, such as cellulose and chitin.

2.2 Determination of carbohydrates

Glucose is the most common carbohydrate classified as a monosaccharide, an aldose, a hexose, and a reducing sugar. Glucose is also called 'blood sugar' as it circulates in the blood at a concentration of 65-110 mg/mL of blood. Glucose is initially synthesized by chlorophyll in plants using carbon dioxide from the air and sunlight as an energy source (equation 2.1). Glucose is further converted to starch for storage in plants. The quantitative analysis of glucose is of relevance not only in medical diagnostics and food analysis due to the importance of glucose concentrations for diabetic patients, but also in bioprocess monitoring, as glucose is the major carbon source in microbial and mammalian cell cultivations. Different methods were developed for glucose determination such as chemical and enzymatic methods, CE, Liquid Chromatography (Shi et al. 2000) or HPLC (Boa et al. 1996). In our study the determination of glucose using enzyme electrodes and microchips capillary electrophoresis was studied.

2.2.1 Enzyme electrodes

2.2.1.1 Definition of biosensors

Since the first enzyme electrode constructed by Clark and Lyons in 1962 using electrochemical detection, the technology of biosensors showed a great development. It became an attractive and powerful tool for biological and biochemical analysis in different fields of application (*e.g.* medicine, agriculture and biotechnology).

A biosensor is defined according to the International Union of Pure and Applied Chemistry IUPAC (Thevenot et al. 1999) as a self-contained integrated device which is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element (biochemical receptor), which is in direct spatial contact with a transducer element. A biosensor should be clearly distinguished from a bioanalytical system, which requires additional processing steps, such as reagent addition. A device which is both disposable after one measurement, i.e. single use, or unable to monitor the analyte concentration continuously should be designated a single-use biosensor.

A physical sensor is defined as a device that detects or measures a physical property and records, indicates or otherwise responds to it.

A chemical sensor is a device that transforms chemical information, ranging from the concentration of a specific sample component to total composition analysis, into an analytical

useful signal. Chemical sensors contain usually two basic components connected in series: a chemical (molecular) recognition system (receptor) and a physico-chemical transducer. Biosensors are chemical sensors in which the recognition system utilizes a biochemical mechanism. The main purpose of the recognition system is to provide the sensor with a high degree of selectivity for the analyte to be measured (Fig. 2.1). In an enzyme biosensor the biological reorganization element is an enzyme (Mulchandani 1998).

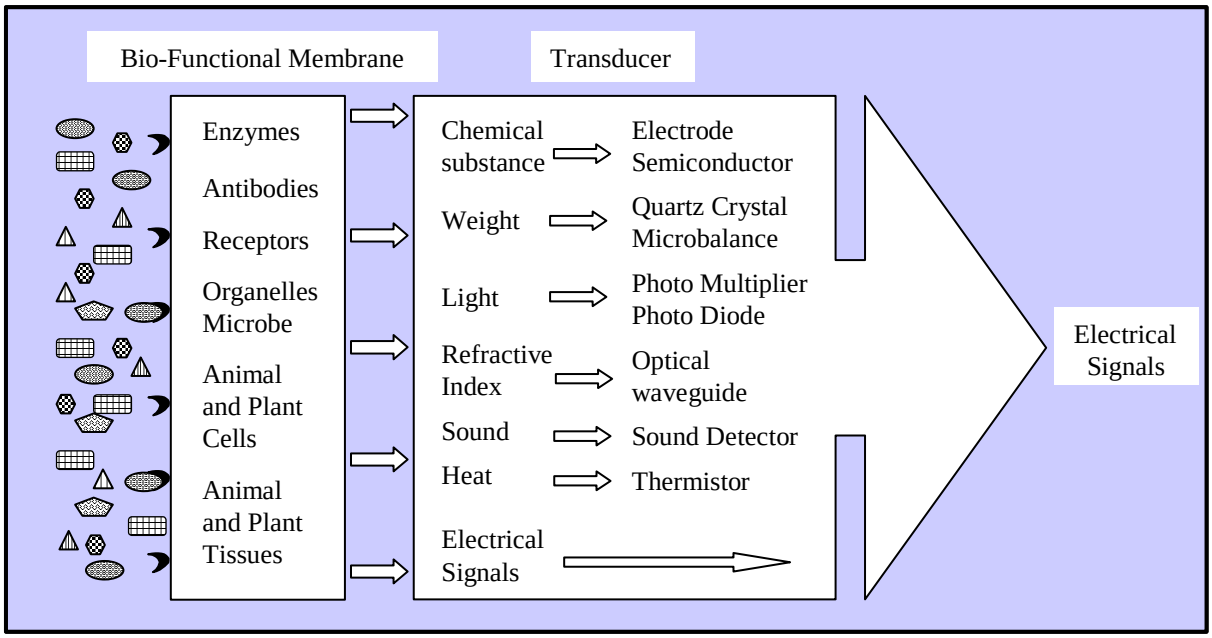


Fig. 2.1 Basic component of biosensors.

2.2.1.2 Transducers

The transducer converts the enzyme reaction into a measurable response, such as current, potential, temperature change or absorption of light through electrochemical, thermal, or optical means.

There are different types of transducers used in enzyme biosensors:

2.2.1.2.1 Electrochemical transducers

An electrochemical biosensor is a biosensor with an electrochemical transducer. According to the transducer used, electrochemical biosensors are divided into several types based on the electrical parameter, which are determined. There are potentiometric, amperometric, and conductometric sensors (Bogdanovskaya et al. 1996; Wang et al. 1997; Zhang et al. 2000; Thevenot et al. 2001; Bakker et al. 2002).

Potentiometric transducers measure the difference in potential, which is generated across an ion selective membrane separating solutions at virtually zero current flow (Mulchandani

1998). Potentiometric devices can measure the changes in pH or ion concentrations. The potential difference is proportional to the logarithm of the concentration of the substance being determined.

Amperometric enzyme biosensors are the most common commercial biosensors available nowadays. This is due to their low cost, rapid response time and higher sensitivity, precision and accuracy compared to potentiometric sensors. In amperometric biosensors the current produced from the electrochemical reaction of an electroactive species at the working electrode is measured as a function of the applied potential. This current is related to the concentration of the species in solution. The selectivity of the amperometric devices is only governed by the redox potential of the electroactive species present; consequently, the current measured by the instrument can include the contributions of several chemical species.

Conductometric biosensors are based on the principle of the change of the conductivity of the solution. This change is due to the production of [electrons or] ions in particular protons or hydroxyl ions from the biochemical reaction.

2.2.1.2.2 Thermal transducers

Thermometric biosensors are based on the measurement of heat evolved or absorbed during the biochemical reaction. The amount of heat evolved or absorbed is related to the molar enthalpy and to the total number of product molecules produced from the biochemical reaction. In thermal biosensors the enzyme is immobilized by cross linking or by entrapment in a membrane close to the thermal transducer (Guilbault et al. 1983), or the enzyme is placed in a temperature-controlled column and the heat produced from the biochemical reaction is measured by recording the temperature difference between the inlet and outlet of the column. Thermal biosensors were used for the determination of glucose in whole blood (Harborn et al. 1997) and urea in serum (Bjarnason et al. 1998). Ramanathan et al. reviewed the development and applications of thermal biosensors for bioprocess monitoring (1999) and the principles and applications of thermal biosensors (2001).

2.2.1.2.3 Optical transducers

Optical biosensors are based on the change in optical properties such as UV-Vis absorption, bio- and chemo luminescence, fluorescence/phosphorescence, reflectance, scattering and refractive index due to the interaction of the biocatalyst with the target analyte (Ramsden 1997; Myszka 1999; Rich et al. 2002).

2.2.1.3 Glucose oxidase (GOD)

Glucose oxidase (β–D-glucose:oxygen 1-oxidoreductase, EC1.1.3.4) from *Aspergillus niger* is a dimer from two identical subunits with a molecular weight of 160 kDa. The dimer contains two disulfide bonds, two free sulfhydryl groups, and two flavin adenine dinucleotide (FAD) molecules tightly bound ($K_a = 1x10^{-10}$) not covalently linked to the enzyme. The flavin cofactors are responsible for the oxidation-reduction properties of the enzyme. The enzyme is glycosylated with a carbohydrate content of 16% (w/w) (Gouda et al. 2003). The protein is readily soluble in 0.1 M potassium phosphate pH 7.0 giving a clear, yellow solution. The native protein is acidic having an isoelectric point (pI) of 4.2. Glucose oxidase catalyses the oxidation of β-D-glucose to D-glucono-1,5-lactone and hydrogen peroxide. This process consists of enzymatic oxidation of glucose by GOD in which the co-factor flavin-adenine dinucleotide (FAD) is reduced to $FADH_2$, followed by the oxidation of the enzyme co-factor by molecular oxygen producing hydrogen peroxide, while the gluconolactone is hydrolysed in aqueous media to gluconic acid according to the following three step reactions:

$$\beta\text{-D-glucose} + GOD(FAD) \rightarrow \text{D-glucono-}\delta\text{-lactone} + GOD(FADH_2) \quad (2.2)$$

$$GOD(FADH_2) + O_2 \rightarrow GOD(FAD) + H_2O_2 \quad (2.3)$$

$$\text{D-glucono-}\delta\text{-lactone} + H_2O \rightarrow \text{D-gluconic acid.} \quad (2.4)$$

The overall reaction is expressed as:

$$\beta\text{-D-glucose} + O_2 + H_2O \rightarrow \text{D-gluconic acid} + H_2O_2 \quad (2.5)$$

GOD has many applications in the food and fermentation industry. It is used also in biosensors technology for medical applications, food analysis and bioprocess monitoring. Immobilization of GOD by cross-linking increased the enzyme stability, where the melting temperature (T_m) increased from 58 ^oC for the soluble enzyme to 76 ^oC after immobilization (Haouz et al. 2001). Various additives such as salts, mono and polyhydroxy alcohols and polyelectrolytes were used to increase the thermal stability of GOD (Gibson et al. 1996; Gouda et al. 2003).

2.2.1.4 Amperometric detection

2.2.1.4.1 Principles

Electrochemistry involves chemical phenomena associated with charge separation at an electrode interface. Voltammetry is one of the electroanalytical methods in which the current of the working (polarized) electrode is measured as a function of the potential applied to the working electrode. Redox reactions occurring at electrodes are heterogeneous and take place in the interfacial region between the electrode and the solution. Amperometry is one of the

voltammetric techniques in which a fixed potential is applied to the working electrode and the current is measured, which is directly related to the electrochemical reaction occurring at the working electrode. At each electrode, charge separation can be represented by the capacitance and the energy barrier for charge transfer as a resistance. The electrode acts as the source or a sink of electrons transferred to or from a molecule at the electrode interface. At steady state the electrode current is limited by diffusion of the substance through the diffusion layer in front of the electrode.

This current can be described by the Fick's first law;

$$i = n\,F\,D\,A\,\frac{C_s}{\delta} \qquad (2.6)$$

where *n* is the number of electrons transferred, *F* is the Faraday constant = 96 487 $Cmol^{-1}$, D the diffusion coefficient of the redox active substance, A the electrode area, δ the thickness of the diffusion layer and C_s the concentration of the substance in the bulk solution.

In amperometric measurements usually a three-electrode set-up is used. The working electrode is the electrode to which the potential is applied and where the relevant oxidation or reduction process occurs. Various materials can be used for creating a working electrode such as carbon, graphite, glassy carbon or inert metals such as gold or platinum. Other metals such as copper, nickel, iridium and rhodium are used for specific applications. The second electrode used in the amperometric cell is the reference electrode, which provides a stable and reproducible potential, against which the potential of the working electrode is compared and defined. The most often used reference electrodes are Ag/AgCl or saturated calomel electrodes SCE (Hg/Hg_2Cl_2). An inert conducting material such as platinum wire or graphite rod is used as the current-carrying auxiliary (counter) electrode. Amperometry is considered as the voltammetric technique most often used and has a wide application in biosensors technology as concentrations of 10^{-8} to 10^{-9} M can be detected (Turner et al. 1987).

Flow Injection Analysis (FIA) is a continuous flow technique, which is ideally suited for rapid automated analysis of liquid samples. This technique was first reported by Ruzicka and Hansen in 1975. FIA is based on the injection of a defined volume of a liquid sample into a moving nonsegmented continuous carrier stream of a suitable liquid. The injected sample forms a zone, which progressively disperses into the carrier and which is transported through the narrow tube under laminar flow conditions towards the detector (Ruzicka and Hansen 1988). The height or area of the peak-shaped signal thus obtained can be used to quantify the analyte after comparison with the peaks obtained for solutions containing known

concentrations of the analyte. Mixing the sample with the carrier stream allows chemical reactions to be performed within the tube. The basic components in FIA systems are a peristaltic pump for the transportation of the carrier through a narrow tube, an injector by which a defined volume of the sample is injected into the carrier stream, valves and a detector (Fig. 2.2).

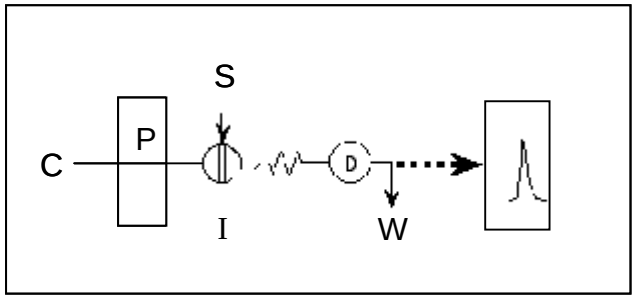


Fig. 2.2 The basic elements in FIA set-up, with S sample, P pump, I injection valve; C carrier solution, W waste, and D detector.

Additional elements can be integrated in the basic FIA set-up such as mixing chambers, catalytic or affinity columns, and gas diffusion or dialysis chambers. FIA is a general solution-handling technique, applicable to a variety of tasks ranging from pH, conductivity, amperometric measurements to colorimetry, titrations and enzymatic assays.

Dispersion is a physical effect related to FIA, where it is defined as the dilution of the injected sample by mixing with the flowing stream due to diffusion and convection processes. The dispersion coefficient (D) is a direct measure for the extent of dilution undergone by a particular part of the sample zone between injection and detection and defined as:

$$D = C_o/C \qquad (2.7)$$

where C_o is the original concentration of the analyte and C is the concentration after transport where dilution occurs. The dispersion coefficient is higher than 1 as C usually is smaller than C_o. The dispersion coefficient is not constant within the sample plug, but reaches a minimum near its centre, where the analyte concentration is maximal. Usually, this value D_{max} is calculated by the following equation:

$$D_{max} = C_o/C_{max} \qquad (2.8)$$

where C_{max} is the maximal concentration reaching the detector (Valcarcel and Luque de Castro 1987).

2.2.1.4.2 Thick film and screen printing technology

The history of thick film technology returned back to 1950s where it was used for the production of hybrid circuits in the field of electronics (Prudenziati 1994). Thick film

technology permits the construction of solid-state, mechanically robust and planar sensors. Glucose (Bilitewski et al. 1991; Silber et al. 1996) and urea (Bilitewski et al. 1992) were detected using biosensors based on thick film technology. The construction of such devices is commonly achieved by the sequential deposition of thick films on a substrate by a screen-printing process (Galan et al. 1995).

Screen-printing techniques is not new, although it is nowadays regarded as a subset of the thick-film technology industry, its historical origins date back some 3000 years to the construction of the Great Wall of China and the manufacture of patterned cloths in ancient Egypt. Since the 1950s it has been applied to the microelectronics industry in the manufacture of printed circuit boards. Thick film technology using a screen-printing procedure is a simple and fast method for mass production of electrochemical sensors. This technique consists of depositing inks of different electrochemical properties on an inert substrate in a film of controlled pattern and thickness. One of the most attractive characteristics of screen-printing technology is its versatility as it is possible to print a variety of different inks (Au, Pt, Ag, *etc.*) on different substrates (such as glass, ceramic and plastic sheet) and to mix modifiers to the base ink.

The basic component of the screen-printing process is shown in Fig. 2.3. The screen is separated from the substrates by a small gap, the substrate is held in its position in the holder. Screen-printing is performed by pressing ink through a screen by means of the moving rubber squeegee. The squeegee brings the screen into contact with the substrate surface dependent on screen tension and squeegee pressure, hardness and speed. The ink is forced through the open areas of the screen by the squeegee leaving behind it a deposit paste as a desired layout. After printing the film is dried (Fig. 2.4) (Alvarez-Icaza and Bilitewski 1993).

In recent years screen-printing technology has gained a great interest in the production of biosensors. The basic steps for screen-printed electrodes are the paste preparation, design and screen production, printing of the paste, drying, firing and encapsulation (Alvarez-Icaza and Bilitewski 1993; Galan-Vidal et al. 1995).

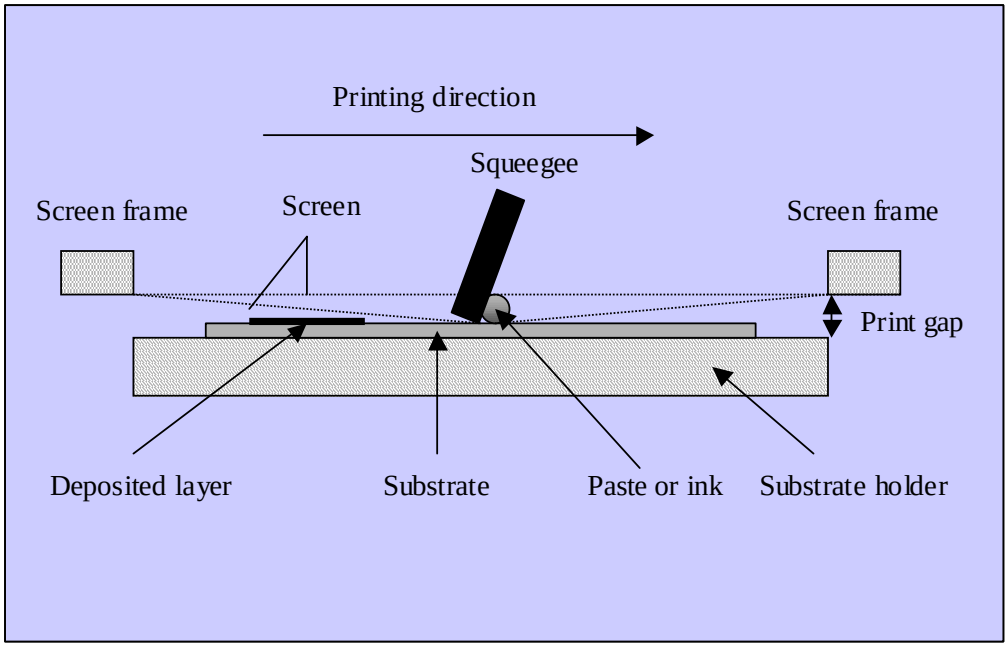


Fig. 2.3 The schematic representation for the screen-printing process.

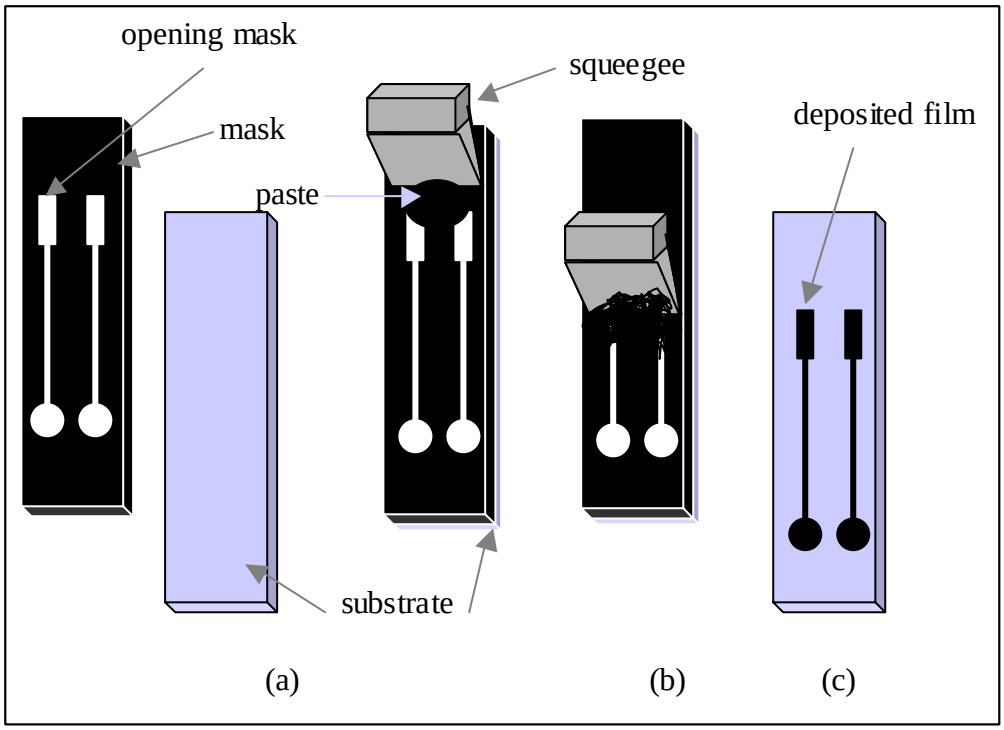


Fig. 2.4 Schematic representation for the screen-printed electrode steps. (a) Mask and substrate preparation before printing; (b) Printing process; (c) Deposited film on the substrate after screen printing process.

First the conducting lines are deposited on the substrate followed by the second step in which the electrodes are printed, and finally the isolating layer is deposited (Fig. 2.5). In each step the ink is deposited in a film of controlled pattern and thickness, followed by drying and firing processes at suitable conditions for polymerization of the ink or paste film.

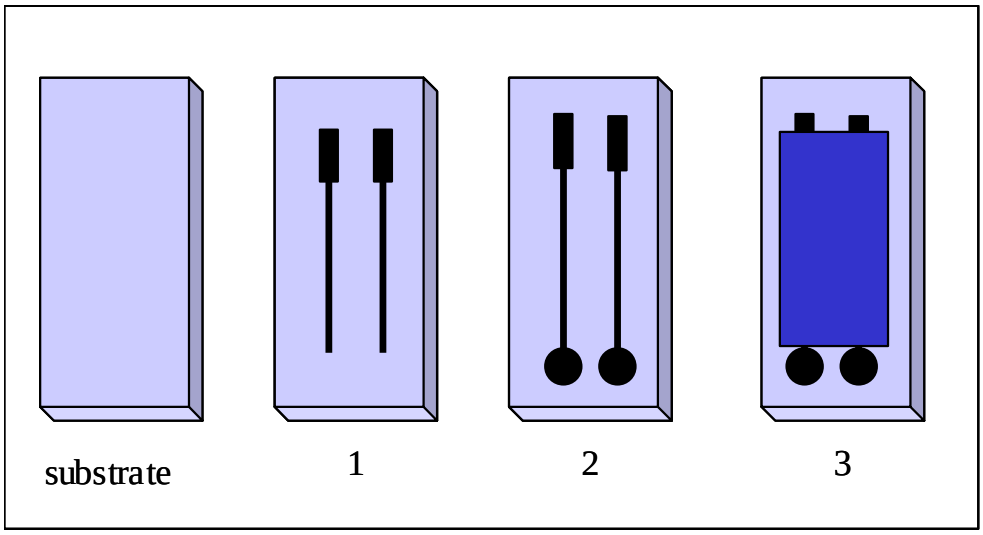


Fig. 2.5 Steps of electrode printing. (1) Printing of the conducting lines; (2) Printing of electrodes; (3) Printing of the isolating layer.

2.2.1.4.3 First generation

In the first generation of biosensors, oxidoreductase enzymes were coupled to amperometric transducers. These sensors were relying on the determination of the product of the enzymatic reactions e.g. hydrogen peroxide, NADH, or on monitoring the consumption of the cosubstrate, e.g. oxygen (Fig. 2.6a).

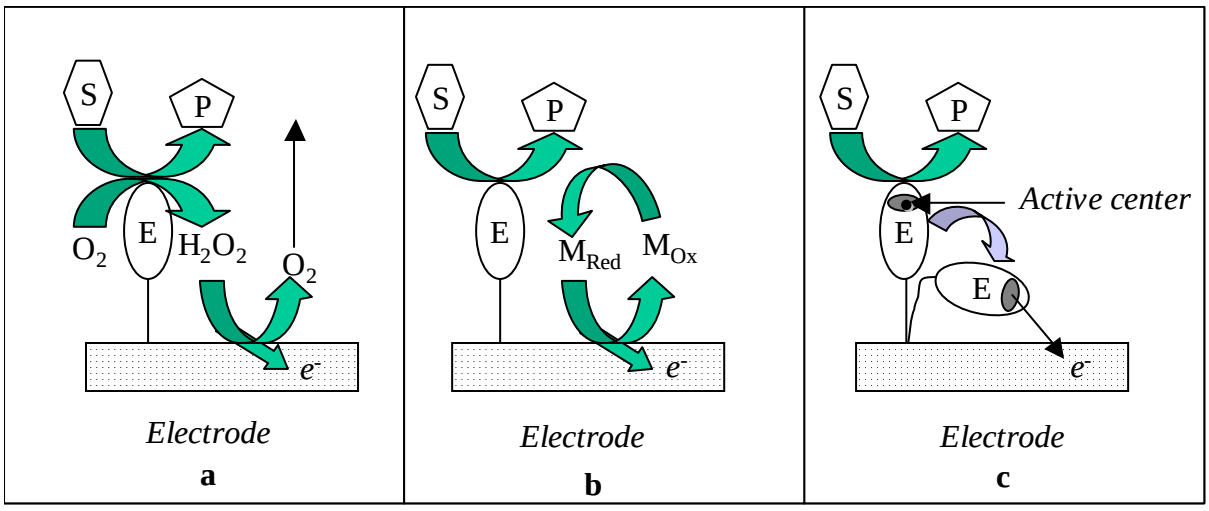


Fig. 2.6 Generations of amperometric biosensors based oxidases: (a) first generation, (b) second generation and (c) third generation. S is substrate, P product, M mediator and E enzyme.

2.2.1.4.3.1 Oxygen detection

The first biosensor introduced by Clark in 1962 consists of soluble glucose oxidase in combination with an oxygen electrode. The oxygen electrode was a two-electrode system, e.g.

a platinum cathode and a silver anode separated from the sample solution by a gas permeable membrane. Oxygen diffusing through this membrane is reduced at the platinum cathode at – 600 mV (vs. Ag/AgCl reference electrode) according to the following equation:

$$O_2 + 2H_2O + 4e^- \rightarrow 4OH^- \qquad (2.9)$$

The resulting current is directly proportional to the partial oxygen pressure and thus indirectly to the substrate concentration.

2.2.1.4.3.2 Hydrogen peroxide detection

Hydrogen peroxide is widely detected in biosensors, as it is produced by oxidases, which can be immobilized directly on the surface of the working electrode. At Pt-electrode, hydrogen peroxide is oxidised at + 600 mV (*vs*. Ag/AgCl reference electrode) according to equation:

$$H_2O_2 \rightarrow O_2 + 2H^+ + 2e^- \qquad (2.10)$$

Hydrogen peroxide can also be detected by the reduction at the working electrode at low potentials i.e. 0.0 V (Pan et al. 2004) as:

$$H_2O_2 + 2H^+ + 2e^- \rightarrow 2H_2O \qquad (2.11)$$

2.2.1.4.3.3 NDAH detection

Glucose dehydrogenase (GDH) catalysis the conversion of β-D-glucose to D-glucono-δ-lactone leading to the oxidation of the cofactor NAD^+.

$$\beta\text{-D-glucose} + NAD^+ \rightarrow \text{D-glucono-}\delta\text{-lactone} + NADH + H^+ \qquad (2.12)$$

NADH can be oxidized at the electrode according to the following equation:

$$NADH \rightarrow NAD^+ + H^+ + 2e^- \qquad (2.13)$$

The oxidation process for NADH requires high overpotentials and the oxidation product causes fouling of the electrode surface. A major problem of NADH- based biosensors is the need to co-immobilize the cosubstrate (NAD^+) within the biolayer. Otherwise NAD^+ will continuously leak from the biolayer with subsequent loss of biosensor stability.

Since almost 40 years specific enzyme electrodes are developed and continuously improved (Wang 2001). They are usually based on glucose oxidase (GOD), an enzyme which catalyses the oxidation of β-D-glucose by dissolved oxygen producing gluconolactone and hydrogen peroxide (equations 2.2 – 2.5). Glucose concentrations can be measured with electrochemical transducers via hydrogen peroxide formation, oxygen depletion or change in pH due to production of gluconic acid. The amperometric detection of enzymatically generated hydrogen peroxide at a polarization voltage of about + 600 mV (*vs.* Ag/AgCl reference electrode) using platinum (Pt) electrodes is the most developed type of glucose biosensors (Xu et al. 2002).

Different electrode materials were used for the electrochemical determination of glucose. Based on thick film technology glucose was detected by immobilization of GOD on a thick film platinum electrodes (Bilitewski et al. 1991; Cui et al. 2000; Muguruna et al. 2000). Combination of amperometric detection with flow injection analysis techniques enables continuous monitoring of glucose in fermentation broth (Huang et al. 1995a; Nandakumar et al. 1999). Detection of H_2O_2 by electrochemical techniques suffers from the interferences from other electroactive compounds present in the sample. Ascorbic acid and uric acid are well-known interferent compounds. To avoid the influence of interferences, immobilization of glucose oxidase e.g. by entrapment in poly-1,3-diaminobenze on a platinum electrode by electropolymerization was performed by Surareungchai et al. 1998. The formed film on the electrode surface improved the selectivity, so that the presence of ascorbic acid gave an error from 6 to 12% using 2 and 8 mmol/L respectively. Rodriguez and Rivas (1999) prepared glucose biosensors by deposition of Iridium and GOD on glassy carbon electrodes. The electrocatalytic action of iridium towards H_2O_2 allowed the determination of glucose at low potentials, which minimize the interferences from ascorbic acid and uric acid. A similar work was reported by Fang et al. (2003) where the effect of interferences was minimized by formation of a monolayer of GOD by covalent immobilization on a semiconducting indium-tin oxide surface. Copper-plated screen-printed carbon electrodes provided a catalytic surface for the amperometric detection of H_2O_2 and avoided interferences by low potentials (Kumar and Zen 2002).

Also to increase the selectivity of glucose biosensors, the addition of Nafion as a negative monomer during the immobilization of GOD prevents interferences from anionic substances such as ascorbic acid and uric acid (Vaidya et al. 1995; Yang et al. 1998; Trojanowicz and Miernik 2001; Xu et al. 2002). Coating of enzyme biosensors by polyion layers of poly-L-lysine and poly(4-styrenesulfonate) were useful to prevent interferences (Mizutani et al. 1998). The polyion complex membrane showed a permselectivity based on the solute size.

2.2.1.4.4 Second generation

In the second generation of amperometric biosensors an electron transfer mediator between the enzyme redox centre and the electrode surface is used (Fig. 2.6b). The mediators may be complexes of transition-metals (*e.g.* hexacyanoferrate (III) and ferrocene, Katrlík et al. 1997), organic substances (*e.g.* catecholamines, Lisdat et al. 1998; *p*-benzoquinone, Casero et al. 2000; Meldola Blue, Avramescu 2002; Vasilescu et al. 2003). Mediators are usually relatively small molecules, which can react with the enzyme redox centre or with a product of the

enzymatic reaction (*e.g.* with H_2O_2, NADH *etc.*) and undergo fast electrochemical redox reactions at relatively low potentials.

The following scheme shows the determination of glucose using a mediator system:

$$\text{glucose} + \text{GOD(FAD)} \rightarrow \text{gluconic acid} + \text{GOD}(FADH_2) \quad (2.14)$$

$$\text{GOD}(FADH_2) + 2M_{(ox)} \rightarrow \text{GOD(FAD)} + 2M_{(red)} + 2H^+ \quad (2.15)$$

$$M_{(red)} \rightarrow 2M_{(oxd)} + 2e^- \quad (2.16)$$

These electrodes are also widespread, because they usually allow the application of less positive electrode potentials and signals are less dependent on oxygen concentrations, the influence of interfering substances can be minimized They are the basis of some commercially available glucose sensors for home monitoring of blood glucose concentrations. Ferrocene and its derivatives are the most commonly used mediators (Nagata, et al. 1995; Ge et al. 1998; Hu et al. 1999; Kulys et al. 2000; Gui et al. 2001; Patel et al. 2003; Ferreyra et al. 2004). Modification of screen-printed electrodes by Prussian blue improved the selectivity and stability of the prepared biosensors (O'Halloran et al. 2001; Ricci et al. 2003). Enzyme electrode based on an inorganic salt $Ru(NH_3)_6^{3+}$ mediator was used for glucose determination (Chen and Gorski 2002). The prepared biosensor showed a high operational stability, no response to interference, a fast response time, and extended long-term storage stability. Electropolymerization of poly(methylene blue) on gold thick film electrode was applied in glucose biosensors (Silber and Hampp 1996). Guemas et al. (2000) prepared a carbon paste electrode with a renewable surface containing GOD, horseradish peroxidase, and ferrocene. In the presence of the soluble enzymes invertase and mutarotase they determined glucose and sucrose in fruit juices using flow injection analysis. In addition to the already mentioned mediators osmium complexes (Zhang et al. 2001), tetrathiafulvalene (Bartlett et al. 1997) and hydroquinone (Wang et al. 1998) were used as a mediators for glucose determination.

2.2.1.4.5 Third generation

Mediated electrodes still suffer from ascorbic acid, uric acid and oxygen interferences, the toxicity of some mediators limits their in vivo applications (Cui et al. 2001; Yang et al. 1998). In addition, a mediated system requires another reagent, which may leak from the sensor and lead to loss in the sensitivity (Kulys 1991). In further developments, electrons are transferred directly between the active centre of the enzyme and the electrode surface (Fig. 2.6c). Only few enzymes are known to allow such behaviour, because the majority of enzymes have the active centre surrounded by a shell of protein. This shell is a serious barrier for the transfer of electrons. However, it is known that horseradish peroxidase can transfer electrons directly

since the redox center is located at the outer part of the proteinic shell (Ruzgas et al. 1996; Varfolomeev et al. 1996; Guo and Guadalupe 1997; Schumacher et al. 2003). The direct electron transfer of immobilized glucose oxidase (GOD) by conversion of GOD($FADH_2$) to GOD(FAD), (where FAD is flavin adenine dinucleotide) was studied. Wang et al. (1994) reported a glucose biosensor by mixing GOD with rhodium-dispersed carbon paste to obtain an intimate contact between the enzyme and carbon particles and achieve direct electron transfer. Pendy et al. (1999) reported enzyme biosensors based on nonmediated and mediated electrochemical oxidation of reduced glucose oxidase encapsulated within organically modified sol-gel glasses electrodes. Recently, Lui and Ju (2003) constructed a reagentless glucose biosensor based on direct electron transfer of glucose oxidase immobilized on colloidal modified carbon paste electrode. Also the conversion of GOD($FADH_2$) to GOD(FAD) was studied at sputtered Pt-electrodes (Os et al. 1996), charge transfer complex electrodes (Khan et al. 1996), carbon paste electrodes (Savitri and Mitra 1998) or colloidal gold modified carbon paste electrodes (Liu and Ju 2003), applying amperometric and cyclic voltammetric techniques. Reagentless glucose biosensors based on GOD entrapped into osmium-complex modified polypyrrole films (Reiter et al. 2001) or based on overoxidized polypyrrole/tetrathiafulvalene-tetracyanoquinodimethane composites (Palmisano et al. 2002; Khan et al. 1996) were reported. The influences of oxygen and other physiological interferences on the glucose response were studied. Uric acid and acetaminophen did not show any significant effect on glucose current, while dissolved oxygen and ascorbic acid showed an effect below 5 and 1% of 10 mM glucose, respectively (Khan et al. 1996), or 0.1 mM ascorbic acid produced a response of ~ 3.1 $\pm$ 1.4% of 5 mM glucose Plamisano et al. 2002).

2.2.1.5 Immobilization principles

Enzymes are proteins that catalyze chemical reactions in living systems according to the general scheme:

$$\text{Substrate} \xrightarrow{\text{enzyme}} \text{products} \qquad (2.17)$$

The enzyme is specific for its substrates, so that only a particular reaction of a particular substrate is catalyzed even in presence of isomers or similar compounds. Enzyme electrodes are based on coupling the enzyme with an appropriate electrode. In such electrodes the combination of the specificity of the enzyme for its substrate with the analytical power of electrochemical devices proved to be extremely useful for monitoring a wide variety of substrates of analytical importance in clinical, environmental, and food samples (Baeumner

2003; Luong 1997; Rogers 1995; Mello and Kubota, 2002; Nakamura and Karube 2003; Keusgen 2002; Bilitewski and Turner 2000; Rogers 1997).

The success of the enzyme electrode depends at least partly on the method of enzyme immobilization. Immobilizing the enzyme on the sensing surface should maintain or even improve the enzyme stability in the enzyme electrode. Immobilisation of enzymes can be achieved by different methods:

2.2.1.5.1 Adsorption

Immobilization of enzymes by adsorption is considered as the simplest method of immobilization, where binding is achieved by just incubating the carrier with the enzyme solution for a sufficient period of time. Binding occurs through hydrophobic interaction, Van der Waals and/or ionic forces (Fig. 2.7a). Various supports are used such as graphite, gold, glass, alumina, cellulose or synthetic polymers.

2.2.1.5.2 Cross-linking

Enzymes can be immobilized to solid supports by cross-linking with low molecular weight bi- or multifunctional reagents producing covalent bonds with intermolecular cross-linking between the reagent and enzyme (Fig. 2.7b). Glutaraldehyde is the bifunctional reagent most often used in the immobilization of proteins, but also other reagents can be taken such as diazobenzidine and its derivatives (Guilbault 1984). The immobilization of enzymes by cross-linking by gluteraldehyde is shown in Fig. 2.8. The activity of the immobilized enzyme depends on different factors such as pH, ionic strength, and number of cross-links produced.

2.2.1.5.3 Adsorption and cross-linking

In this type of immobilization the enzyme is adsorbed to the solid support followed by cross-linking with glutaraldehyde (Fig. 1.7c). This method is rapid and simple and the immobilized enzyme retained its activity and was stable (Weetall 1974).

2.2.1.5.4 Covalent bonding

Enzymes can also immobilized by the covalent bond between the functional groups of the enzyme and the support matrix (Fig. 2.7d). In this method nucleophilic groups present in amino acid side chains of proteins are used for coupling. These groups include NH_2, CO_2H, OH, C_6H_4OH, SH, and imidazole. Reactions need to be performed under mild conditions such as low temperature, low ionic strength and pH in the physiological range. To protect the

active site, the reaction is often carried out in the presence of the enzyme substrate (Turner et al. 1987).

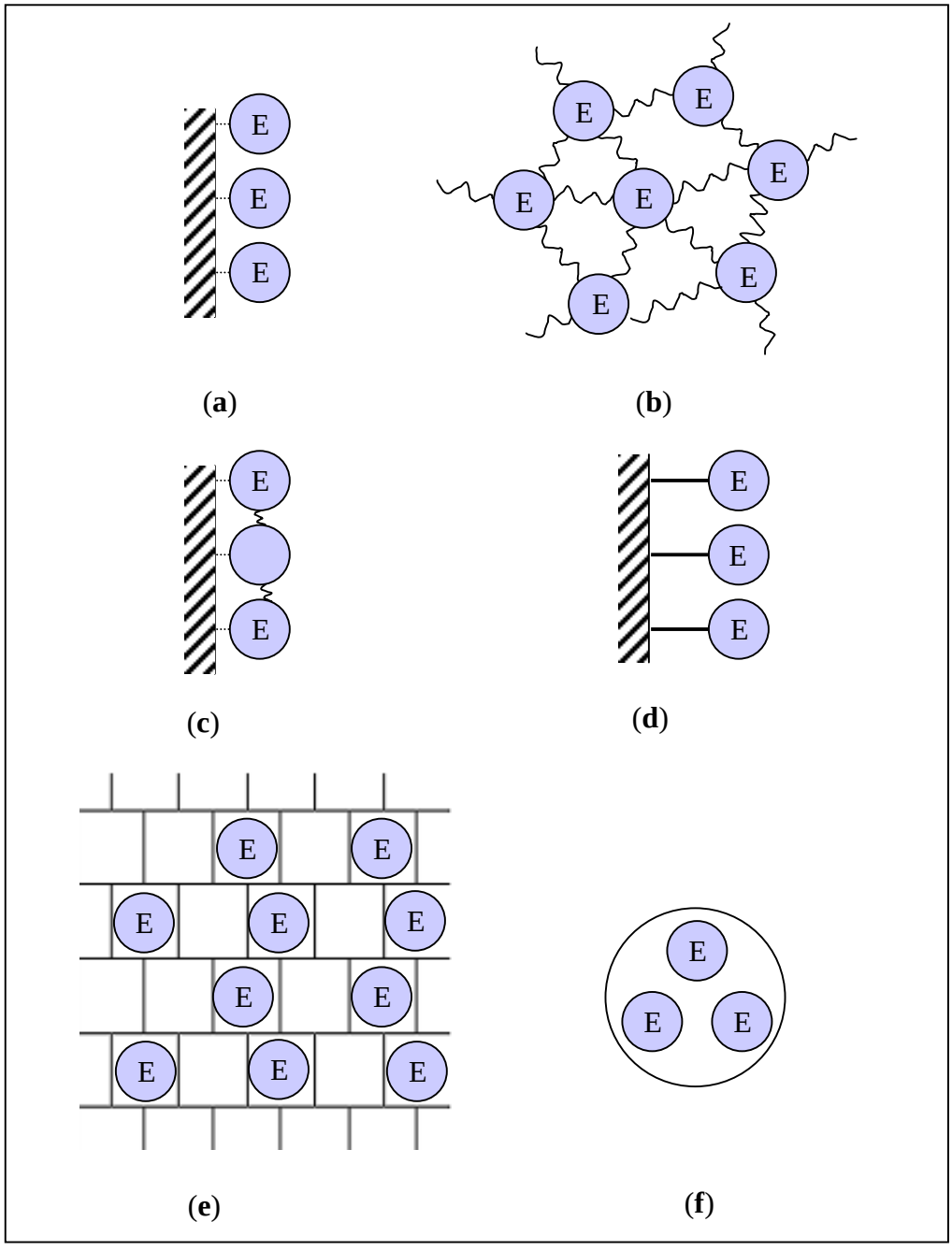


Fig. 2.7 Different types of enzyme immobilization: (a) adsorption; (b) cross-linking; (c) adsorption followed by cross-linking; (d) covalent bonding; (e) entrapment; (f) encapsulation.

2.2.1.5.5 Entrapment

Enzymes can be entrapped within the lattice of a polymer matrix or a membrane (Fig. 2.7e), so that the proteins are retained while substrates are allowed to penetrate. This method differs from the covalent binding and cross-linking in that the enzyme itself does not bind to the gel

matrix or membrane. The most common polymer used is polyacrylamide. It is prepared by copolymerisation of acrylamide with N, N'- methylenebisacrylamide. Other materials used are starch gels, nylon and conducting polymers such as polypyrrole.

2.2.1.5.6 Microencapsulation

In this method of immobilization an inert membrane is used to trap the enzyme on the transducer (Fig. 2.7f). The main types of membranes used are cellulose acetate, polycarbonate, collagen and polytetrafluroroethylene (PTFE). Nafion or polyurethane can also be used.

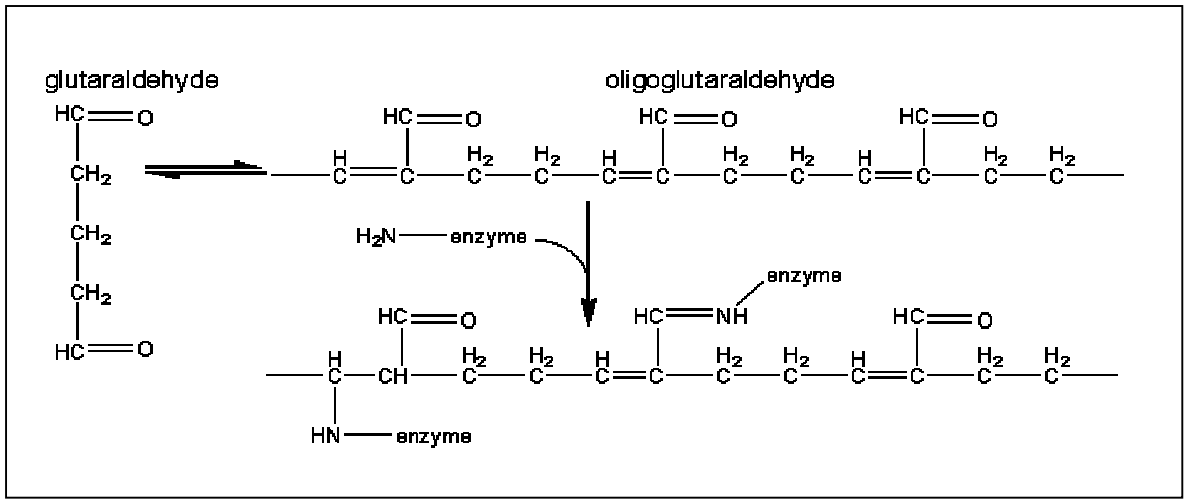


Fig. 2.8 Immobilization of enzyme by cross-linking using the bifunctional reagent glutaraldehyde.

2.2.1.6 Stability of enzyme electrodes

Stability of enzymes is one of the important considerations for applications of enzyme electrodes. The choice of the immobilization method has a great effect on the stability of the immobilized enzyme. In order to increase the stability of immobilized enzymes different additives were added to the enzyme during the immobilization procedure. The addition of polymers, such as diethylaminoethyl (DEAE)-dextran and the non-reducing sugar alcohol, lactitol, increased the stability of enzymes. This was probably due to interactions between the additive material and water molecules surrounding the enzyme structure, which reduced the water activity and increased intermolecular hydrophobic bonding of the polypeptide chains of the protein. More energy is needed to disrupt the folded structure of the protein (Gibson et al. 1992a; Gibson et al. 1992b; Gibson et al. 1996; Appleton et al. 1997; Gavalas et al. 1998; Gavalas and Chaniotakis 2000). Addition of Gafquat enhanced the shelf life of biosensors due to the formation protein-polyelectrolyte complexes (Gibson et al. 1996). Additionally,

chemical contributions from side-chains were discussed (Wimmer et al. 1997). Other polymers such as poly(*O*-phenylenediamine) or Nafion led to a good stability of the enzyme (Krawczynski vel Krawczyk et al. 1996; Trojanowicz and Mirnik 2001). The effect of enzyme immobilization in carbon paste electrodes in the presence of solid additives such as zeolite 6 and chemical additives such as cationic polymers or cationic, low molecular weight polyhydroxyls and lactitol on the stability and selectivity of enzymes was studied by Lutz et al. (1995). An improvement of the direct electron transfer of the catechol/*o*-quinone couple was obtained with both solid and chemical additives included in the carbon paste and also the sensitivity was improved by the addition of chemical additives while solid additives showed a negative effect. Recently protein based stabilizing agents (PBSA) such as lysozyme, BSA and gelatin were used in combination with glutaraldehyde for enzyme stabilization (Gouda et al. 2000; Gouda et al. 2002). The stability of enzyme immobilized on screen-printed electrodes was improved by mixing DEAE-dextran, hydroxyethyl cellulose or co-polymer of vinyl pyrrolidone and dimethylaminoethyl methacrylate and polyethyleneimine with the sugar alcohol, lactitol (Hart et al. 1995; Hart and Collier 1998; Schumacher et al. 1999)

2.2.1.7 Selectivity of enzyme biosensors

Selectivity is another important characteristic of enzyme electrodes. Amperometric biosensors suffer from the effect of interferences i.e. other electrochemically active compounds, which are present in a sample together with the analyte. Various strategies were developed for increasing the selectivity of enzyme electrodes. One useful strategy is the employment of a permselective coating that minimizes the access of oxidizable compounds to the transducer surface by allowing only the transport of the analyte (Wang and Lu 1990; Sasso et al. 1990; Malitesta et al. 1990; Wang 1991; Moussy et al. 1993; Zhang et al. 1994; Vadgama 1997). Reduction of the applied electrode potential using mediators (2^{nd} generation of biosensors) (Schumacher et al. 1999; Murthy et al. 1998; Wang et al. 1999; Chen et al. 2002; O'Halloran et al. 2001) is another alternative to reduce the influence of interferences. Mediated electrodes still suffer from ascorbic acid and uric acid interferences, the toxicity of some mediators limits their in vivo applications (Cui et al. 2001; Yang et al. 1998) and the loss of sensitivity due to the loss of the amount of mediator limits sensor stability (Morales et al. 1996).

However, it is increasingly recognized that additives influence additional properties of enzyme electrodes. Thus, improvements of direct electron transfers between the protein and the electrochemical transducer (Popescu et al. 1995; Liu and Deng 1995) and increases in signals were observed for different enzymes (Schumacher et al. 1999; Schumacher et al. 2001). Occasionally, charged polymers are used to reduce the accessibility of charged

interferents to the electrode, thus improving selectivity (Yang et al. 1998; Xu and Yu 2002; Karyakin et al. 1996; Trojanowicz and Miernik 2001; Liu and Deng 1995; Vaidya et al. 1995; Pan and Arnold 1996). Cellulose acetate membrane was used to increase the selectivity of microbial biosensors for determination of ethanol during fermentation (Tkac et al. 2003; Reday and Vadgama 1997).

2.2.1.8 Miniaturised enzyme assays

In chemical analysis of different analytes very small volumes are required due to the miniaturization of enzyme electrodes for inset in Microsystems. Frebel et al. (1997) were constructed a microelectrode array, which consists of 400 microelectrodes with 36 x 36 µm size for each electrode. This microelectrode array was used for the determination of glucose, L-lactate and uric acid after immobilization of a mixture of enzymes. Different microelectrodes were developed for glucose, lactate and pyruvate with different electrode diameter ranging from 1.0 to 1.2 mm (Schaffar 2002) or 50 to 500µm (Revzin et al. 2002). Miniaturization of enzyme electrodes enables to use them in capillary electrophoresis or in microchips for electrochemical detection. Application of electrochemical detection of carbohydrates using enzymatic reactions in capillary electrophoresis or microchips gained little attention till now. This may be due to the adsorption of enzyme onto the inner surface of the capillary, which reduces the sensitivity, or reproducibility. Protein adsorption usually reduces the electroosmotic flow (EOF) so that migration times of analyte increase leading additionally to reduced peak heights and increased peak widths. Thus, sensitivity and reproducibility of the assay decrease with repeated experiments. One single example for determination of glucose was studied by Wang et al. (2000). Where glucose was detected using GOD and electrochemical detection of peroxide with a screen-printed carbon band working electrode. Separation of glucose from uric acid and ascorbic acid was also examined. In our work, determination of glucose using enzymatic reaction in fused silica capillaries and different kinds of microchips was studied. The effects of different additives to eliminate protein adsorption on the inner wall were examined.

2.2.2 Capillary electrophoresis

2.2.2.1 Fundamentals of Capillary electrophoresis

Capillary electrophoresis (CE) has wide spread application in analytical and bioanalytical chemistry. The applications of CE in pharmaceutical, biomedical, clinical, environmental analysis and in biotechnology have been reported in several review articles (Lunte and O'Shea, 1994; Wang and Fang 2000; Hempel 2003; Wätzig and Günter 2003; Fu et al. 2003;

Petersen et al. 2003; Pico et al. 2003; Issaq 2000; Kennedy 1999; Rassi 1999; Frazier et al. 1999; Lagu 1999; Nishi 1999; Deyl et al. 1998; Altria et al. 1998; Lehmann et al. 1997; Jenkins and Guerin 1996; Paulus and Klockow 1996; Malik and Faubel 2001).

Electrophoresis is defined as the migration of charged ions either by attraction or repulsion in an electric field. Practically a negative electrode (cathode) and a positive electrode (anode) are placed in a solution containing ions. When the electric field is applied, cations (positive) migrate towards the cathode and anions (negative) are attracted towards the anode. Neutral solutes are not attracted to either electrode. Conventionally electrophoresis has been performed on layers of gel or paper. Detection of the separated bands was performed by post-separation visualisation. The analysis times were long as only relatively low voltages could be applied before excessive heat formation caused loss of separation.

The application of electrophoresis in capillaries was accomplished by Hjerten et al. (1965, 1967) using 3 mm tube for the analysis of different analytes such as inorganic ions, nucleotides, proteins or viruses. Virtanen (1974) used a 0.2 mm capillary tube to eliminate the convection problems. In the late 1970s/early 1980s, application of electrophoresis on narrow tubes (75 µm) had become viable (Mikkers et al. 1979; Jorgenson and Lukacs 1981, 1983).

Performing electrophoretic separations in capillaries has many advantages as it offers the possibility of automated analytical equipment, fast analysis times and on-line detection of the separated peaks. Heat generated inside the capillary is effectively dissipated through the walls of the capillary, which allows high voltages to be used to achieve rapid separations.

The simplified schematic capillary electrophoresis set-up is shown in Fig. 2.9. It consists of the following main parts:

1- Capillary (coated by polyimide) with a very small inner diameter (i.d. 10-50µm).
2- High voltage power supply.
3- Two electrodes made of an inert material such as platinum for the connection of the high voltage from the source to the reservoirs.
4- Detection system.
5- Two reservoirs, that can accommodate both capillary and electrodes connected to the power supply.

In capillary electrophoresis the electric field is used to move a sample through a capillary filled with electrolyte solution, which conducts current through the capillary. The ends of the capillary are dipped into reservoirs filled with the electrolyte. Electrodes made of an inert material such as platinum are also inserted into the electrolyte reservoirs to complete the electrical circuit. A small volume of sample is injected into one end of the capillary. An area

at the other end of the capillary is designated as the detection window through which molecules are detected by a variety of techniques.

The plot of detector response with time is generated which is termed an electropherogram. A flow of electrolyte, known as electroosmotic flow, EOF, results in a flow of the solution along the capillary usually towards the detector.

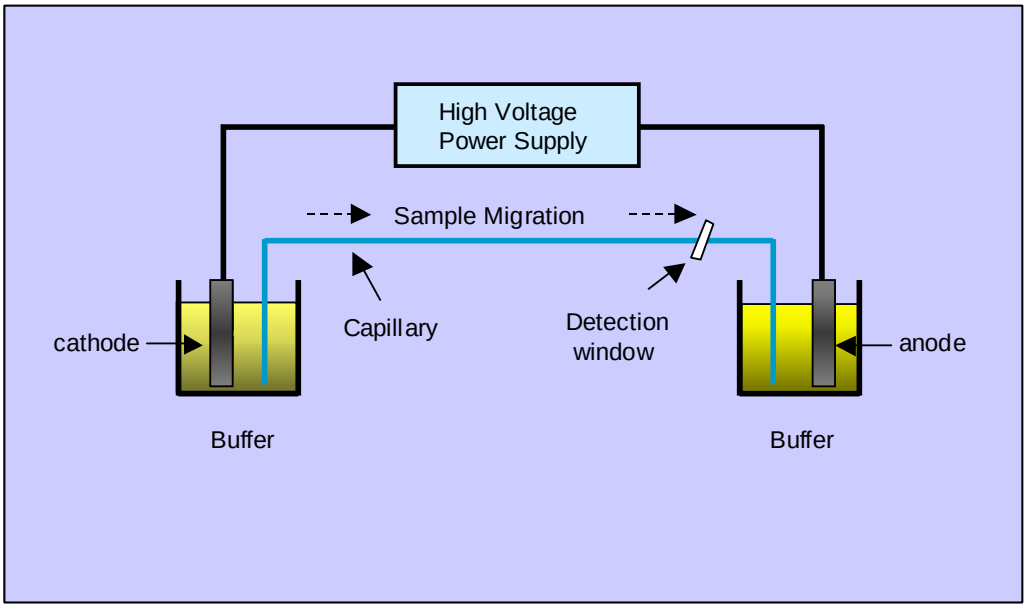


Fig. 2.9 Schematic capillary electrophoresis set-up.

2.2.2.2 Electrokinetic Mobility

In capillary electrophoresis molecules are transported by two principles. The first is movement due to the electric field, the resulting flow rate is called electrophoretic velocity. The second is the bulk flow of the solution due to the surface charge on the capillary wall, which called electroosmotic flow (EOF).

2.2.2.2.1 Electrophoretic Mobility

In electrophoresis, the electrophoretic velocity (v) of ions increases by increasing the electric field strength (E). The electrophoretic mobility μ_e of ions relates to the electrophoretic velocity v and the electric field strength E by the following equation:

$$\nu = \mu_e E \qquad (2.18)$$

The velocity of an ion is determined by dividing the effective capillary length L_{eff} (from the inlet to the detector) by the migration time t_m.

$$\nu = \mu_e E = \frac{L_{eff}}{t_m} \qquad (2.19)$$

The electrophoretic mobility μ_e indicates how fast ions move through a given medium. It is a balance of two forces acting on the ions, the electrical force F_e acts in favour of motion and the fractional force F_f acts against motion.

The electrical force is given by:

$$F_e = q\,E \qquad (2.20)$$

where q is the charge of the ion.

Stokes' Law gives the fractional force

$$F_f = -\,6\,\pi\,\eta\,r\,\nu \qquad (2.21)$$

where η is the dynamic viscosity (Pa s) and r the Stokes radius (cm).

At steady state the two forces are equal, but in opposite directions:

$$q\,E == 6\,\pi\,\eta\,r\,\nu \qquad (2.22)$$

Combining equation 2.18 with 2.22 leads to the following equation for the electrophoretic

$$\mu_e = \frac{q}{6\,\pi\,\eta\,r} \qquad (2.23)$$

It depends on the charge and the ion radius (size), ions with higher charge and small size will have a higher mobility than ions with lower charge and larger size. Due to differences in the charge to size ratio for different ions they have different migration velocities in the same electric field strength and therefore they are separated. The mobility can be calculated from the electrophoretic experiment using the following equation:

$$\mu_e = \frac{L_{eff}\,L_t}{V\,t_m} \qquad (2.24)$$

where L_t is the total capillary length and V the applied voltage.

2.2.2.2.2 Electroosmotic Flow (EOF)

Fused silica capillaries contain silanol groups, which are dissociated, depending on the pH, giving a negative surface charge. When the capillary is filled by a background electrolyte, the cationic components will be attracted to the negative charged wall forming a double layer (Fig. 2.10). In the double layer positive ions predominate and are arranged in a rigid and diffuse layer. The first layer formed closest to the wall is called Stern layer and is tightly bonded to the capillary surface by the electrostatic forces. Some ions diffuse into the solution due to thermal motion forming the second layer, which is called the diffuse layer (Stevens and Cortes 1983). According to the charge distribution the resultant potential is divided into two

regions, a linear decrease of the potential in the rigid layer (a) and an exponential decrease in the diffusion layer (b). The latter is known as Zeta-potential (ζ).

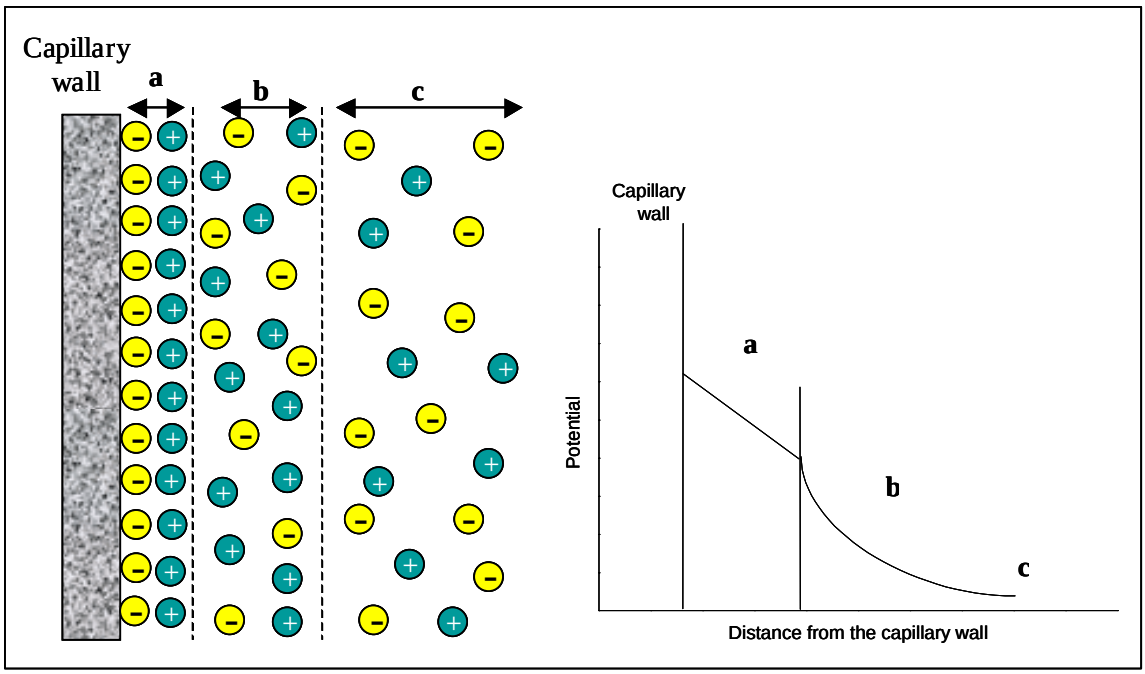


Fig. 2.10 Schematic of double layer charge distribution at a negatively charged capillary wall and formation of the ζ–potential. (a) Rigid boundary layer with adsorbed layer; (b) Diffusion layer and (c) Electrolyte.

The ζ-potential is affected by the surface charge and the ionic strength of the background electrolyte. If an electric field is applied, the cations in the diffuse layer are free to migrate towards the cathode, carrying the bulk solution with them. The result is a net flow of the buffer in the direction of cathode, causing the EOF. Its mobility is directly proportional to the ζ-potential and the dielectric constant (ε) of the background electrolyte and inversely proportional to the viscosity of the medium (η):

$$\mu_{EOF} = \frac{\varepsilon \zeta}{4 \pi \eta} \qquad (2.25)$$

For fused silica capillaries the ζ-potential is proportional to the charge density on the capillary wall surface, which in turn is dependent on the pH of the buffer. At higher pH the EOF will be significantly greater than at low pH. At pH > 9 the silanol groups are completely ionised and the EOF is greatest, while at pH < 4 the ionisation of silanol groups is low and the EOF mobility is insignificant. The ζ-potential is dependent also on the ionic strength of the buffer, by increasing the ionic strength the double layer will be compressed, which decreases the ζ-potential and the EOF.

The migration velocity of the electroosmotic flow (ν_{EOF}) depends on the electric field strength, *E*, and is given by the Helmholtz equation:

$$\nu_{EOF} = \mu_{EOF} E = \frac{\varepsilon \zeta E}{4 \pi \eta} \qquad (2.26)$$

In the presence of EOF the mobility of compounds is called the apparent mobility (μ_a) which includes both the electrophoretic mobility (μ_e) and the electroosmotic mobility (μ_{EOF}):

$$\mu_a = \mu_e + \mu_{EOF} \qquad (2.27)$$

The electroosmotic flow can be determined by injecting an uncharged substance (neutral marker), for which is $\mu_e = 0$. Thus, the velocity and mobility determined via the migration time are only due to the EOF (equations 2.19 and 2.25) (Huang et al. 1983).

The EOF can be significantly greater than the electrophoretic mobility of the individual ions contained in the sample. Consequently, both anions and cations can be separated and determined in the same run. Cations are attracted towards the cathode and their speed is increased by the EOF. Although anions are electrophoretically attracted towards the anode, they are carried towards the cathode with the electroosmotic flow of the buffer. Cations with the highest charge/mass ratios will migrate first, followed by the cations with reduced ratios. Next, neutral components migrate with the same velocity as the EOF, and finally, the anions migrate. Those anions with lower charge/mass ratios migrate faster than those with greater ones (Fig. 2.11).

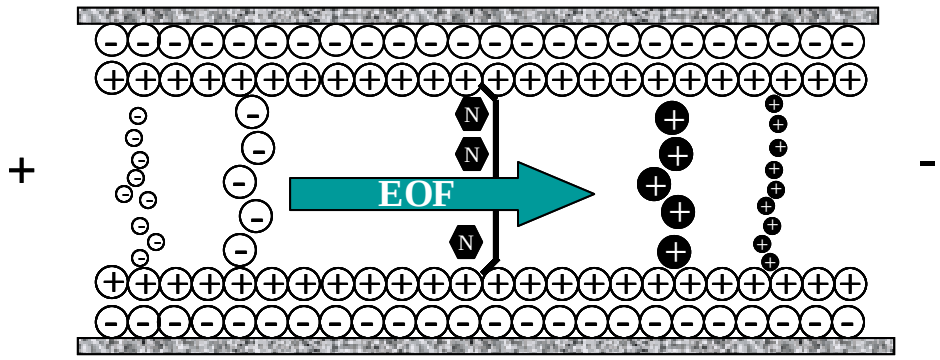


Fig. 2.11 Electroosmotic flow in uncoated fused silica capillary.

One of the advantages of electrosmotic flow is the flat flow profile, which is shown in Fig. 2.12. This is in contrast to that produced from external pumps leading to pressurized flow, which yields a parabolic, or laminar flow as used in HPLC. The flat flow profile produced from EOF is due to the driving force from the charge on the capillary wall, which is uniformly distributed along the capillary. So there are no pressure drops and the flow velocity is uniform

across the capillary cross section. This contrasts with flow due to using pressure (in HPLC), in which the frictional force at the column wall causes a pressure drop across the column, yielding a parabolic flow profile.

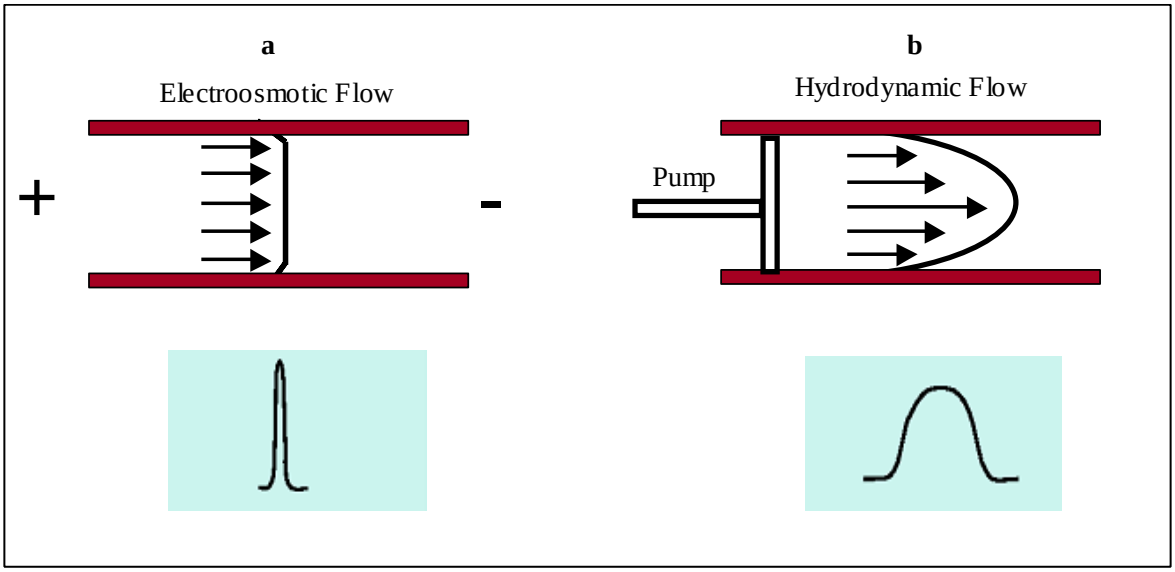


Fig. 2.12 Flow profile of EOF (a) and laminar flow (b).

The flow profile obtained in capillary electrophoresis by EOF is important because it minimizes band broadening, which enhances the separation efficiency.

In normal capillary electrophoresis or in capillary electrophoresis microchips, the transportation of neutral or negatively charged analyts towards the cathode depends on the electroosmotic flow (EOF) created from the presence of negatively charged silanol groups on the inner surface of glass capillaries. If proteins are components of the capillary electrophoretic assay it is known that they tend to adsorb onto the inner surface of the capillary due to electrostatic interactions between positively charged residues on the proteins and the negatively charged silanol groups of the capillary surface (Cifuentes et al. 1996). This results in changes of the surface charge and typically to a reduction of the EOF, loss of separation efficiency (Lauer and McManigill 1986), poor reproducibility of migration time (Hjerten and Kubo 1993) and low protein recovery (Towns and Regnier 1991).

In order to minimize the adsorption of proteins and to obtain a stable EOF, different approaches have been described in the literature. One strategy is the use of electrolyte solutions with extreme pH values with untreated capillaries because at low acidic pH-values the dissociation of silanol groups is suppressed (McCormick 1988), while at pH values higher than the protein pI both protein and the inner capillary surface are negatively charged (Lauer and McManigill 1986). This, however, is not feasible in all applications, in particular if functional, active proteins are required. Instead capillaries can be used, of which the surface is

coated permanently with a not-charged polymer so that the EOF but also the electrostatic adsorption of proteins are suppressed. These coatings, however, show a limited long-term stability and are not easily applicable to microchips. Thus, frequently dynamic modifications are performed, i.e. the running buffer is supplied with additives such as polymers (Verzola et al. 2000; Cordova et al. 1997; Erim et al. 1995; Gonzalez et al. 2003), salts, amines, zwitterions, or surfactants (Laueret al. 1986; Quang et al. 2003; Green and Jorgenson 1989; Bushey and Jorgenson 1989). Surfactants are of particular importance, as they are available with different charged groups and can form micelles, if the concentration is sufficiently high. The anionic surfactant sodium dodecylsulfate (SDS) was extensively used in protein separation, where it enhances the EOF by increasing the density of negative charges on the inner capillary surface (Miksik et al 2002; Miksik and Deyl 1999; Badal et al. 2002). Neutral surfactants such as Tween (Towns and Regnier 1991) or Brij 35 (Badal et al. 2002) are also used as a capillary surface coating to prevent the adsorption of proteins. However, most frequently cationic surfactants are used as buffer additives. Addition of cationic surfactants above the critical micelle concentration (CMC) leads to electrostatic adsorption on the negatively charged capillary wall and formation of a double-layer so that the wall surface is finally positively charged and the EOF reversed. The positive charge on the capillary surface electrostatically repels positively charged basic proteins and thus decreases adsorption. Several cationic surfactants were used, such as tetradecetyltrimethylammonium bromide (C_{14}TAB), cetyltrimethylammonium bromide (C_{16}TAB) and didodecyldimethylammonium bromide (DDAB) (Badal et al. 2002; Lucy and Underhill 1996; Melanson et al. 2000; Baryla et al. 2001; Yeung et al. 2001; Baryla and Lucy 2002).

2.2.2.3 Separation modes in capillary electrophoresis

2.2.2.3.1 Capillary Zone Electrophoresis (CZE)

In CZE the running buffer (also called the background electrolyte) is flushed through the capillary by mean of pressure and used also to fill the inlet and outlet reservoirs. The sample is injected at the inlet end of the capillary and the high voltage is applied. The components migrate towards the detection point (outlet) according to their mass-to-charge ratio.

CZE is the most widely used technique in capillary electrophoresis, as it offers several advantages in comparison with other CE separation modes. CZE is a simple technique where a single buffer is used in the capillary and electrode reservoirs (Fig. 2.13). The selectivity (i.e. the separation efficiency) depends on the pH, type, concentration and composition of buffer, capillary length, and the applied high voltage (Engelhardt et al. 1994).

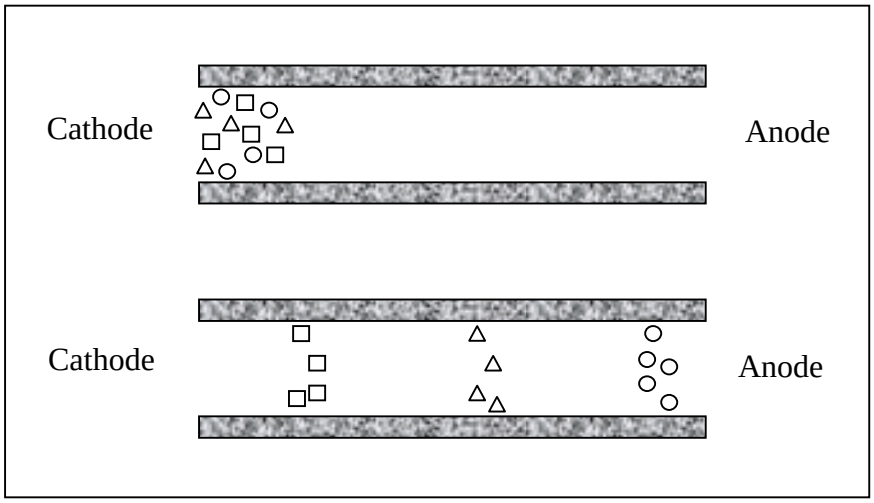


Fig. 2.13 Schematic representation of capillary zone electrophoresis (CZE).

2.2.2.3.2 Capillary Gel Electrophoresis (CGE)

In this technique the capillary is filled by a gel matrix, which acts as a molecular sieve for separation of compounds based on size. CGE is applied to the separation of macromolecules such as proteins or nucleic acids, which possess the same mass–to-charge ratio with different size and can not be separated in a electric filed. The gel affects the mobility of large molecules more than of smaller ones (Fig. 2. 14). The bigger molecules will be slowed down more than the smaller ones by the gel and migrate later through the capillary (Engelhardt et al. 1994; Marina and Torre 1994).

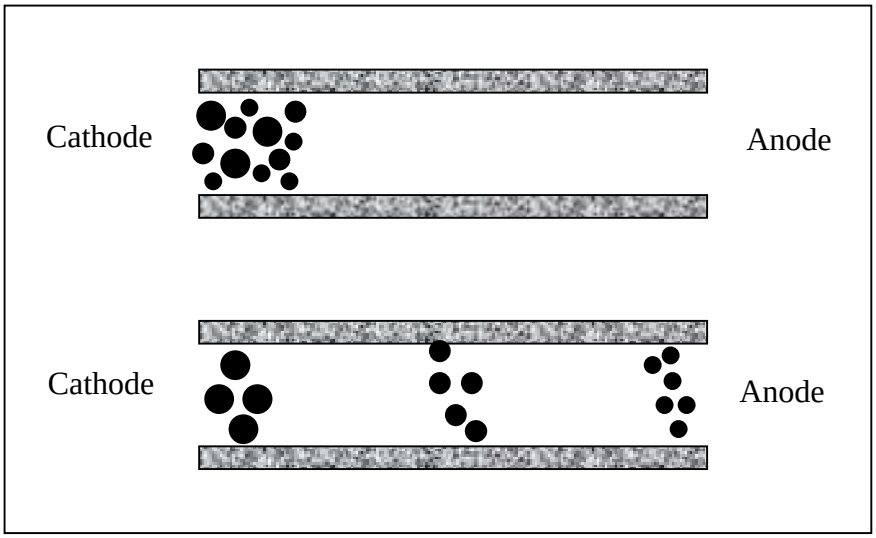


Fig. 2.14 Schematic representation of the separation on macromolecules based on molecular size using capillary gel electrophoresis (CGE).

2.2.2.3.3 Capillary Isoelectric Focusing (CIEF)

In CIEF amphoteric analytes (which have both acidic and basic behaviours such as peptides and proteins) are separated based on their isoelectric points (pI) at which they have a net zero charge, thus they are not electrophoretically mobile. For electrophoretic separations based on the pI a pH gradient covering the range of pIs is desired. To generate the pH gradients, a solution of amphoteric substances (such as aliphatic aminocarboxylic acids) whose pI values cover the pH range is placed in the capillary. Ampholytes carry a positive or a negative charge depending on the pH value. A strong acid is placed in the anode and a strong base is placed in the cathode vial and an electric filed is applied. Protons and hydroxide ions begin to flow and the ampholytes are arranged according to their pIs building up pH gradients. Proteins will migrate to the pH value that equals their pI value. Thus, different proteins will migrate to a different position in the capillary forming focused zones (Fig. 2.15). These zones are passed to the detector either mechanically by application of a pressure difference (pressure or suction) to the system or breaking down the pH gradients without loss in the separation and regain the mobility of the focused analytes. This can be achieved by adding higher concentrations of salt ions to the detector side such as sodium ions at the anode for anode mobilization or chloride ions at the cathode for cathode mobilization (Engelhardt et al. 1994; Marina and Torre 1994). CIEF is a very sensitive technique with a good resolution, short time of analysis and very small amounts of analyte required.

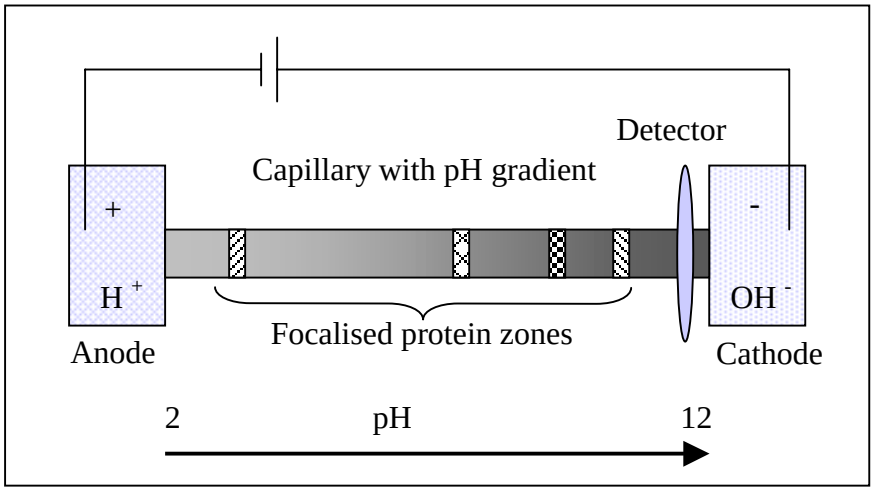


Fig. 2.15 Schematic representation of capillary isoelectric focusing (CIEF).

2.2.2.3.4 Capillary Isotachophoresis (CITP)

In CITP the sample is injected into the capillary between two electrolytes with different ion mobilities, one is a leading electrolyte (LE) with ion mobility greater than protein components and the second is the terminating electrolyte (TE) with an ion mobility lower than protein

components (Fig. 2.16). At steady state, the ions with the same charge will migrate with the same rate forming different zones, which migrate at equal velocity towards the detection point as a step with zone length proportional to the concentration (Engelhardt et al. 1994; Wehr et al. 1999). ITP is not used as a separation method because no peaks are obtained but step diagrams. The zones follow each other directly and are not separated. Each zone contains a single compound. CITP is used as preconcentration techniques for dilute solutions, where very narrow sample zones of a high concentration can be obtained.

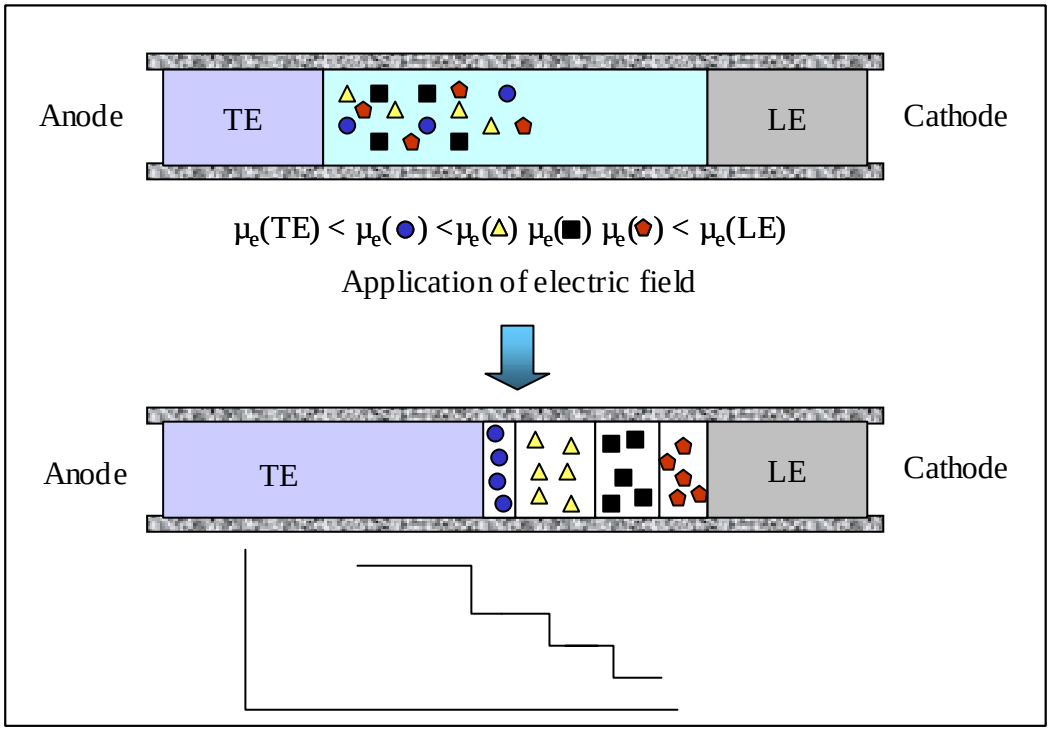


Fig. 2.16 Schematic representation of capillary Isotachophoresis (ITP).

2.2.2.3.5 Micellar Electrokinetic Chromatography (MEKC)

In this technique a higher concentration from an ionic surfactant above its critical micelle concentration (CMC) is added to the background electrolytes. Sodium dodecyl sulfate (SDS) is the most often used surfactant in MEKC. The surfactant monomers form spherical aggregates called micelles in which the hydrophobic parts are oriented to the centre of the micelle and the charged or polar groups are oriented to the surface of the micelle. Since the surfaces of the SDS-micelle have a negative charge, the electrophoretic mobility is oriented to the anode, while the EOF is directed towards the cathode. The sample molecule will distribute between phases, the bulk aqueous mobile phase and the surfactant micelles, depending on their hydrophobic character (Fig. 2.17). More hydrophobic compounds have higher retention times. Cationic surfactants, non-ionic and zwitterionic surfactants have been also used (Engelhardt et al. 1994; Wehr et al. 1999).

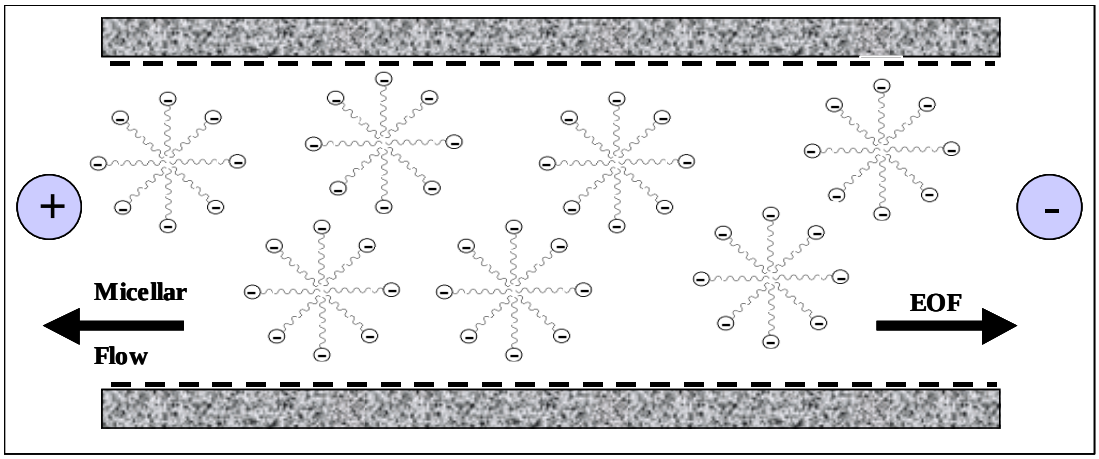


Fig. 2.17 Schematic representation of Micellar Electrokinetic Chromatography (MEKC).

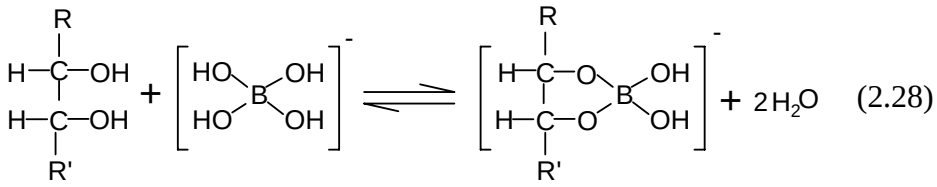
2.2.2.3.6 Capillary Electrochromatography (CEC)

In capillary electrochromatography, the capillary is packed with standard HPLC packing materials such as porous silica beds with a large negative surface charge. The surfaces of silica beds have sufficient densities of ionised silanol groups to generate a high EOF, which drives the separation, when the electric field is applied to the system (Wehr et al. 1999). Due to the different flow profiles obtained in CE and HPLC (see Fig. 2.12) a reduced band broadening is expected in CEC systems.

Determination and separation of carbohydrates using capillary zone electrophoresis are based on either by enzymatic assays or direct transportation and detection using different detection methods. Determination of glucose using enzymatic reactions was reported by Frerichs and Colon (1998) using laser-induced fluorescence (LIF). The enzymatic produced H_2O_2 reacted with the flourogenic reagent in the presence of peroxidase to form a fluorescent product, which is proportional to glucose concentration.

Electrophoretic separations are based on different mobilities of sample components in an electrical field. Except MEKC and CEC, which are based on differences in partition and chromatographic interactions, all CE modes can separate only require charged solutes. Some carbohydrates derivatives possess charged functional groups in their structures, such as aldonic acids, uronic acids, sialic acids, amino sugars (glucosamine, galactosamine), and sulfated sugars, which allow their electrophoretic migration in an electric field (El Rassi 1995). For the separation of neutral carbohydrates by electrophoresis the conversion of these polyhydroxy compounds into a charged species is required. Several strategies were used to charge the neutral carbohydrates, these strategies include:

Tetrahydroxy borate ion $B(OH)_4^-$ can form complexes with polyhydroxy compounds under alkaline conditions according to the following mechanism (El Rassi 1995):



(2.28)

The sugar-borate complexes as seen from equation 2.28 are negatively charged and under normal EOF conditions, with normal polarity would migrate against the direction of the EOF.

Carbohydrates form a complex with some alkaline earth metal cations such as Ca^{2+}, Pb^{2+}, Zn^{2+}, etc. The formed complexes were separated from each other by the differences of their capability of complexation using capillary electrophoresis (Honda et al. 1991; El Rassi 1995).

Another method used for the determination of carbohydrates was the derivatization with a charged label. In this method the CE label is used to introduce both charge and optical properties to carbohydrates to allow electrophoretic separation and sensitive UV and LIF detection (Lamari et al. 2003).

The hydroxyl groups of carbohydrates are ionized under extreme alkaline pH, forming negatively charged alcoholates. The ionization constants for carbohydrates are very low and in the range of 10^{-12}-10^{-14}, i.e. pK_a = 12-14. Thus carbohydrates behave as very weak acids in strong alkaline solutions having pHs > 11. Under these conditions carbohydrates were separated by capillary zone electrophoresis (El Rassi 1995). NaOH 0.05-1.0 M is the electrolyte solution most often used for the separation of carbohydrates. Separation of carbohydrates at extreme alkaline pH can be combined with four different detection methods: indirect UV detection (Lee and Lin 1996; Liu et al. 1997; Soga and Imaizumi 2001; Soga and Serwe 2000; Zeman et al. 1997), refractive index detection (Ivanov et al. 2000) or mass spectrometric detection (Klamfl et al. 2001) and electrochemical detection (Voegel and Baldwin 1997).

Amperometric detection is the electrochemical technique most often used for the detection of carbohydrates by capillary electrophoresis using different working electrode materials such as Au, Pt, Cu, and Ni. Usually, the pulsed amperometric detection (PAD) mode is required to obtain stable and reproducible results (O'Shea, and Lunte 1993; Lu and Cassidy 1993; Weber and Lunte 1997). PAD with Au or Pt electrodes avoid fouling of the electrode surface by carbohydrate oxidation products. For Ni or Cu electrodes no potential pulsing is required (Colon et al. 1993; Ye and Baldwin 1993; Huang and Kok 1995; Fermier et al. 1996; Hu et al. 2002; Hua et al. 2000; Hua and Tan 2000; Hu et al. 2001; Goto et al. 2001). Glucose, fructose, and sucrose were simultaneously detected by capillary electrophoresis using amperometric detection at a disk-shaped copper electrode where 0.2 mM CTAB was used to reverse the EOF (Fu et al. 1998).

2.2.2.4 Microchips

Miniaturization of analytical devices is increasingly attracting interest in analytical chemistry. The driving force is the demand for the production of low cost instruments capable of analysing compounds in relatively short times using small sample volumes with a high level of automation. In combinatorial chemistry large numbers of compounds are produced in single synthesis runs and rapid screening methods are required for analysing and characterizing small amounts of product (~10^{-12} L). In pharmaceutical analysis or drug discovery fast screening methods are needed for characterization of new drug candidates. In medical diagnostics, beside the need to use very small volumes, also the decrease in the analysis time is required, where the time factor may signify a fatal issue for the patient. Also in DNA sequencing, polymerase chain reactions (PCR) and in the synthesis and characterization of libraries of peptides and oligonucleotides high sensitivities and selectivities are required. The high cost of the chemicals in many pharmaceutical and biochemical studies increases the demand for the miniaturization (Manz and Becker 1998). Capillary electrophoresis microchips are a very important step towards the miniaturization of analytical separation methods.

In 1979, Terry et al. reported a miniaturized gas chromatograph air analyser fabricated on a silicon wafer. This was considered the first analytical microdevice reported, since this time microdevices did not receive much interest until the introduction of the µTAS or lab-on-a-chip-concept by Manz et al. in the early 1990s. Several books and reviews introduced µTAS systems and covered their theory, technology and applications in chemistry and life science (Manz and Becker 1998; Reyes et al. 2002; Aruoux et al. 2002). Microfluidic devices showed several applications in different fields such as biochemical analysis (Kopp et al. 1997; Bilitewski et al. 2003; Khandurina and Guttman 2002), clinical, pharmaceutical and bioanalytical research (Verpoorte 2002; Huikko et al. 2003; Gawron et al. 2001; Sandres and Manz 2000; Dario et al. 2000; Aruoux et al. 2002; Jakeway et al. 2000).

2.2.2.4.1 Microchip materials and fabrication techniques.

Since the introduction of lab-on-a-chip devices in 1990s, glass (pyrex or borosilicate) has been the dominant substrate material for fabrication (Manz et al. 1992; Harrison et al. 1992; Fan and Harrison 1994). Quartz was also used as substrate for microchips (Jacobson et al. 1995; Suzuki et al. 2003; Tachibana et al. 2003), while the use of silicon (Terry et al. 1979; Manz et al. 1990; Harrison et al. 1993) was limited due to its conductive behaviour at high voltages needed (Mognesen et al. 2001).

The microfabrication process (Manz et al. 1992) for glass microchips includes four different groups of processes: deposition, photolithography, etching, and bonding (Fig. 2.18):

1- Deposition of a metal film (usually chromium with a thin gold layer, inorganic oxides e.g. SiO_2, or polymers to promote adhesion) followed by a photoresist layer.
2- Photolithography to transform a layout from a photomask (which is produced with a standard lithography process and contains the structural information of the device) to a photoresist film by using UV, X-ray or electron beam lithography.
3- Etching procedures to form microchannels on the substrate, including wet or dry isotropic or anisotropic etching. Wet isotropic etching involves aqueous solutions from hydrofluoric acid (HF) and nitric acid, and wet anisotropic etching involves aqueous basic solutions containing KOH or tetramethyl ammonium hydroxide (Fintschenko and van den Berg 1998; Jackeway et al. 2000). The composition of the solution determines the final shape of the channel. Isotropic etching occurs at the same rate in all directions (Fintschenko and van den Berg 1998) yielding a sloping wall and relatively low aspect ratios features, while the anisotropic etching process yields vertical walls with higher aspect ratios (Jackeway et al. 2000). Dry etching processes such as reactive ion etching (RIE) or advanced silicon etching (ASE) are performed by using a plasma etching machine (Fintschenko and van den Berg 1998; Becker and Gärtner 2000).
4- The final stage of the microfluidic chip fabrication is the bonding step, which includes closing the channel and preparation of the required reservoirs, using low-temperature processes. Anodic bonding is used to bond glass to a silicon substrate by electrostatic attraction (by application of high positive DC voltage) to form covalent bonds. For quartz glass thermal bonding (450-900 ^oC) is used to assemble the substrates. Fusion bonding is used to bond glass-glass (fused silica) at high temperature (< 1000 ^oC) without any intermediate layers. HF bonding is also used to assemble glass-glass. In this process after cleaning of the substrates by acetone, methanol, and H_2SO_4 and H_2O_2, the top substrate is placed on the bottom substrate and 1% of HF solution is dropped into the gap between the substrates and spreads. Pressure is applied to obtain reliable bonding (Shoji 1999).

Glass is the most common material for µTAS devices not only due to the well-established process but also due to the generation of the electroosmotic flow and its chemical stability towards aqueous or non-aqueous solvents. One of the drawbacks of glass is that their products are mechanically fragile and the biocompatibility is relatively poor. Biomolecules (such as

oligonucleotides, DNA or proteins *etc*.), have hydrophobic, ionic, and electrostatic interactions with fused silica surfaces, which can result in the adsorption of compounds and degradation of the separation efficiency (Cobb et al. 1990). This problem can be prevented by a surface coating as mentioned later. The use of harmful wet chemistry (e.g. HF) in different steps in the fabrication process (cleaning, resist coating, photolithography, development, wet etching) and the limitations in geometrical design due to the isotropicity of etching processes, which allow only semicircular channel cross sections in glass substrates, are other disadvantages for using glass in microfluidic chips (Becker and Gärtner 2000).

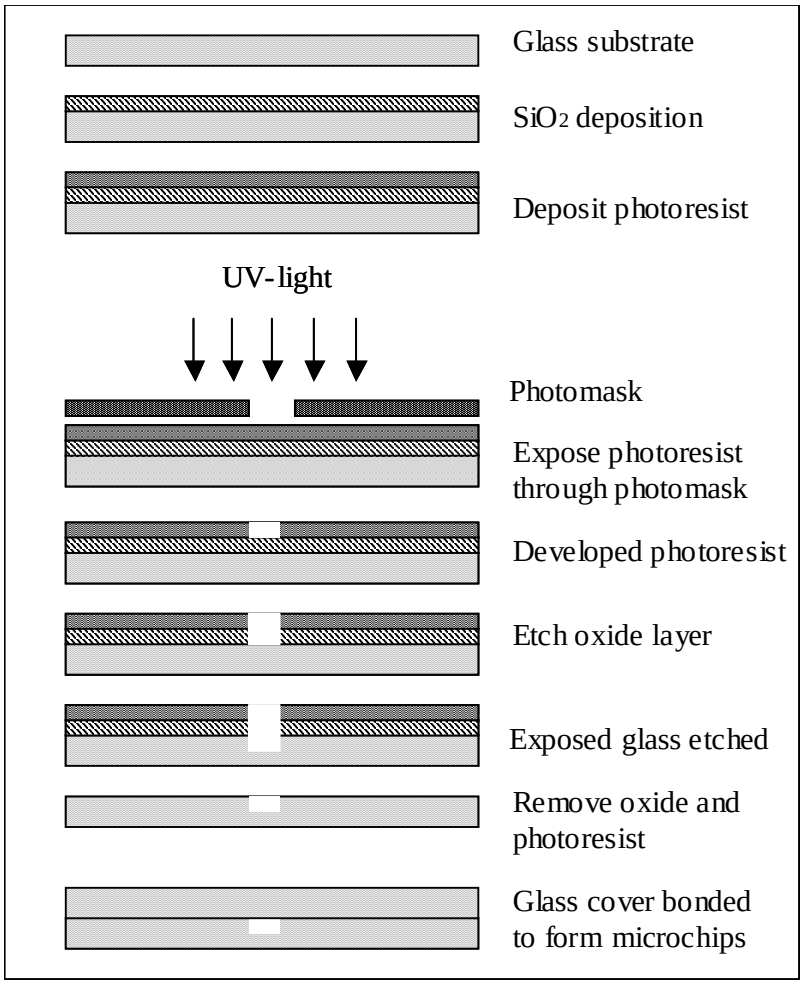


Fig. 2.18 Fabrication procedure for glass microchips.

Fabrication of microchips using polymer materials attracted wide interest in the last years because they are inexpensive, easy to handle, ready to accept casting, molding and machining procedures. Moreover a wide variety of polymer materials can be used for microfluidic chips fabrications. The most popular polymer materials are polymethylmethacrylate (PMMA), poly(dimethylsiloxane) (PDMS) and polycarbonate (PC) with different methods of fabrication which include imprinting, laser micromachining, injection molding, casting, milling, hot

embossing X-ray and ultraviolet polymer lithography (Martynova et al. 1997; Lee et al. 2001; McCormick et al. 1997; Wang et al. 2002; Graß et al. 2001; Soper et al.1999; Chen et al. 2000; McDonald et al 2000, Rossier et al. 2002; Becker and Gärtner 2000; Gawron et al. 2001; McDonald et al. 2000; Fujii 2002; Becker and Locascio 2002; Geschke et al. 2004).
Fabrication of microfluidic chips from ceramic material (aluminium borosilicate ceramic by Low-Temperature Co-fired Ceramic LTCC) was also reported (Henry et al. 1999).

2.2.2.4.2 Injection modes in CE microchips

In microchips there are two types of microchips: microchips with T-intersection (tee-injector design) or double T-intersection (double tee-injector). In microchips with T-intersection it is difficult to control the sample plug, so this type of microchips is rarely used and replaced by the double T-intersection. In microchips with double T-intersection, the sample is introduced by three different injection modes: unpinched injection, pinched, and gated modes. If amperometric detectors are used, the detection reservoir (called also buffer waste BW), which contains the working, reference, and auxiliary electrodes, must be held at ground. This not required when optical detection is used and a voltage can be applied to this reservoir without affecting the detector response.

2.2.2.4.2.1 Unpiched injection (or Floating injection)

In this type of injections only a single power supply is required. A high voltage is applied to the SR for 1-5 s with the sample waste reservoir (SW) grounded (Fig 2.19a), and buffer reservoir (BR) and sample waste (SW) floating. In the separation step the voltage is applied to BR, BW grounded and both SW and SW are floating. In unpinched injection there is no pushback voltages used to keep the sample from leaking into the separation channel (Wang et al. 1999; Martin et al. 2000).

2.2.2.4.2.2 Pinched injection

In this type of injection, the sample is introduced by application of voltage at the SR, while SW is grounded and a voltage is also applied at both BR and BW (Fig 2.19b). During separation high voltage is applied to BR to prevent sample leakage and a fraction of this high voltage is applied to both SR and SW, and BW is grounded (Jacobson et al. 1994; Zhang and Manz 2001). The application of lower high voltage to SR and SW maintains a small pushback flow in these channels and keeps the analyte from leaking into the separation channel while maintaining the desired plug shape.

2.2.2.4.2.3 Gated injection

In gated injection, the sample is introduced by electrophoretic migration along the separation channel (not as in the pinched mode in which the sample is loaded in the injection cross). The sample is placed at the head of the separation channel and the buffer placed in one of the side reservoirs. The sample is introduced by application of a high voltage at BR and a fraction of this voltage is applied to SR, while SW and BW (detection) are grounded. In this way the sample flows towards the SW and BW reservoirs (Fig 2.19c). Gated injection is accomplished by floating the voltage at the BR for a short period of time. This sweeps sample into the separation channel and the separation is initiated by resuming the high voltage to the BR (Liu et al. 2000). The amount of sample introduced in the chip by gated injection depends on the electric field and the applied time.

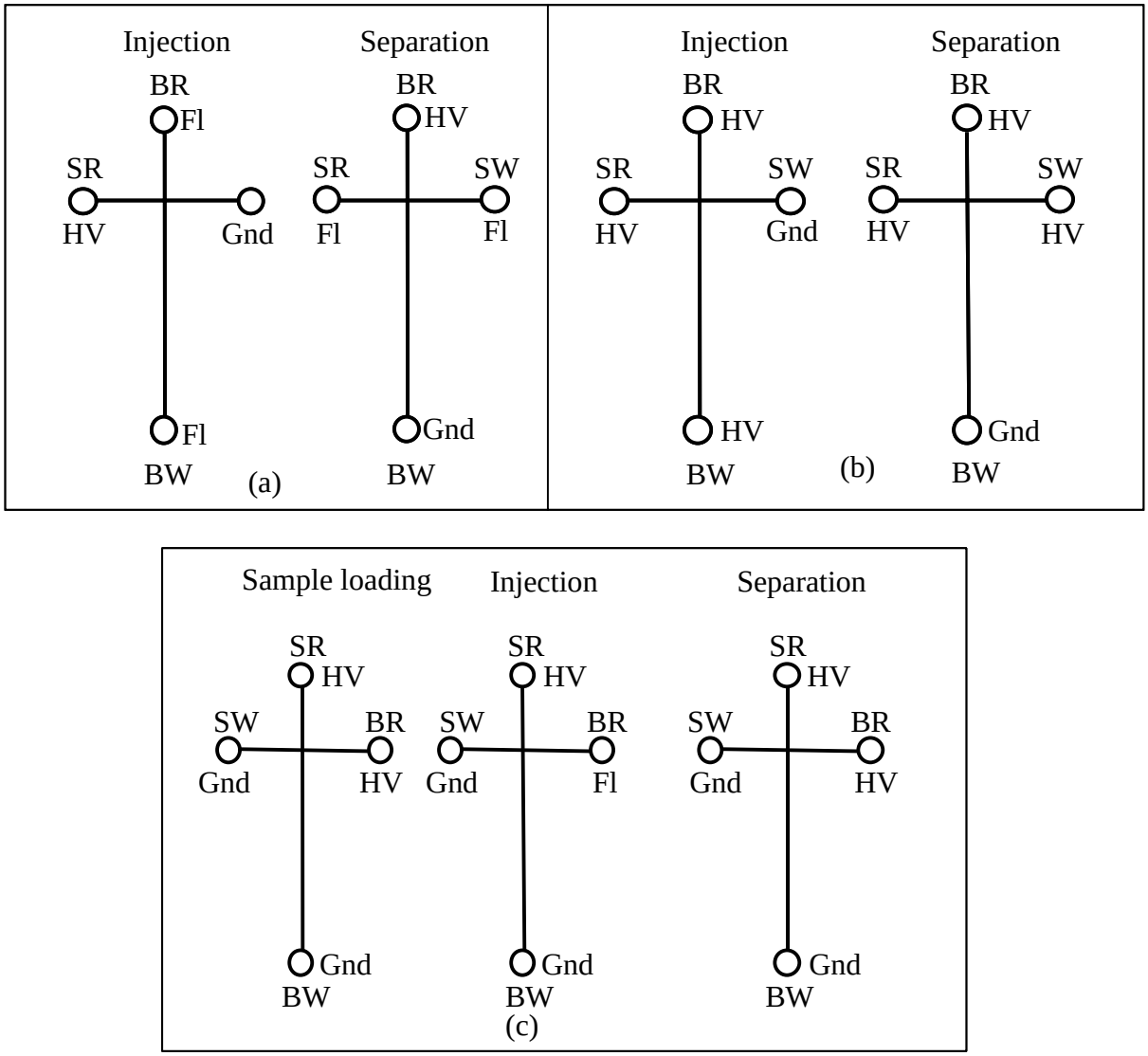


Fig. 2.19 Injection modes used in capillary electrophoresis microchips: (a) unpinched injection; (b) pinched injection; (c) gated injection.

2.2.2.4.3 Detection methods in capillary electrophoresis microchips

Different detection methods are used in microchip capillary electrophoresis. Laser-induced fluorescence (LIF) is the most popular optical method used due to its sensitivity and the ease of focusing the laser directly into the small separation channel (Jacobson et al. 1994; Suzuki et al. 2001; Munro et al. 2000; Qin et al. 2004). LIF faced some disadvantages where it requires a post-capillary derivatization of the sample with a fluorophore and the limited number of wavelengths, which can be used for excitation. Mass spectrometry (MS) has been also employed for the detection in microchips, as it is considered a powerful tool in analytical chemistry (Figeys and Aebersold 1998; Ramsey and Ramsey 1997; Xue et al. 1997; Tachibana et al. 2003). Mass spectrometry gives more chemical information than other detection methods but the available systems are more costly and less sensitive than LIF. Electrochemical detection is the most attractive technique for microchips due to its high sensitivity and selectivity. Frequently, it can be used without derivatization of analytes and it is not expensive like the other detection methods. Different types of electrochemical detection modes were used such as conductimetric detection in which the conductance of the solution is measured and the resultant response is proportional to the analyte concentration (Baldwin 2000; Tanyanyiwa et al. 2002; Tuma et al. 2002; Lichtenberg et al. 2002; Tanyanyiwa and Hauser 2002). Potentiometry is another electrochemical technique used in microchips, in which the potential difference established across an ion selective membrane is measured and gives information about the analyte composition (Baldwin 2000; Tanyanyiwa et al. 2002). Sinusoidal voltammetry was also used as a detection technique in microchip electrophoresis devices (Herbert et al. 2002 and 2003). Amperometric detection is the electrochemical technique most widely used in microchips. A fixed potential is applied to the working electrode and the resultant current from the reduction or oxidation of the analyte is measured. Several recent work employed amperometric detection in microchips capillary electrophoresis (Lacher et al. 2001; Vandaveer IV et al. 2002; Wang 2002; Backofen et al. 2002; Wang et al. 2000; Fanguy and Henry 2002). In recent years the fabrication of microchips with integrated electrodes for conductivity detection (Graß et al. 2001; Lichtenberg et al. 2002) and for amperometric detection was reported by several groups (Wooly et al. 1998; Wang et al. 1999; Wang et al. 1999; Baldwin et al. 2002; Herbert et al. 2003; Wilke and Büttgenbach 2003; Erickson and Li 2004).

2.2.2.4.4 Influence of high electric field on amperometric detection

Although amperometric detection in microchips spreads it is faced with interferences from the high electric field used for the separation of compounds. No significant effects from the high electric field on the amperometric detection was observed if the capillary had a very small inner diameter (≤ 25µm). Using capillaries with very small inner diameters reduced the separation current and caused the separation field rapidly to decay at the end of the capillary (Huang et al. 1991; Lu and Cassidy 1994). To eliminate or avoid the influence from the electric field on the detection if the capillary is larger several approaches were used. The first approach used is the end-channel detection, in which the working electrode is placed tens of micrometers from the end of the separation channel. At this distance the separation voltage is effectively isolated from the working electrode, as it rapidly drops at the end of the capillary (Huang et al. 1991; Wallenborg et al. 1999). Using end-channel amperometric detection capillaries with 25 and 50 µm i.d. can be used without any electrical field decoupler, but a shift for the applied potential on the working electrode due the presence of the electric field was observed (Matysik 1996). The second approach is in-channel detection, in which the working electrode is placed directly within the separation channel and the analyte migrates over the working electrode. This eliminates band broadening (Martin et al. 2002). The last approach used is off-channel detection, in which the position of the working electrode is similar to the In-channel detection, but the separation voltage is isolated from the detection by using a decoupler (Chen et al. 2001; Osbourn and Lunte 2003; Rossier 2000).

2.2.2.4.5 Carbohydrate detection by microchips capillary electrophoresis

In all examples microfluidic structures made in poly(dimethylsiloxane) (PDMS) were used. Detection was based on the electrochemical methods such as PAD at a Pt- (Fanguy and Henry 2002) or a Au-electrode (Garcia and Henry 2003) or sinusoidal voltammetry at a planar copper electrode (Herbert et al. 2002), or on indirect fluorescence detection with 0.1 mM disodium fluorescence added to the background electrolyte (Monahan et al. 2002). Using alkaline conditions glucose, fructose and sucrose (Monahan et al. 2002) or glucose and xylose or sucrose, respectively (Herbert et al. 2002) were separated. With sodium tetraborate glucose and mannose were separated (Garcia and Henry 2003).

3 AIM OF THE THESIS

The general aim of the presented work was the elimination of matrix effect in the electrochemical determination of carbohydrates.

The specific determination of a single carbohydrate, in particular of glucose is achieved by enzyme assays, i.e. assays based on glucose oxidase. Thus, the composition of the immobilization matrix used for the production of screen-printed glucose electrodes was to be optimised to eliminate the interference from ascorbic acid, which was used as model interferent. Different types of additives such as cationic polymers (poly-L-lysine or Gafquat 755N), negative polymer Nafion, neutral polymer polyethylene glycol (PEG), graphite powder and the negative surfactant sodium dodecyl sulfate (SDS) were examined.

As an alternative approach capillary electrophoresis in microchips was studied. Different microchip materials such as glass, poly(methylmethacrylate) (PMMA) and ceramic were investigated. These materials were chosen because glass microchips are already commercially available and PMMA and ceramic microchips were produced in industrial processes.

To increase the separation efficiency in microchip capillary electrophoresis three different strategies were used. The first one was the modification of the inner capillary surface using cationic and anionic surfactants to eliminate enzyme adsorption and get a stable electroosmotic flow (EOF). The second strategy was the immobilization of glucose oxidase (GOD) in the capillary of microchips for glucose detection. Different types of immobilization methods with different materials were to be examined using fused silica capillaries and glass microchips. Also the capillary electrophoretic separation combined with the amperometric determination of some carbohydrates using different types of background electrolytes in the CE procedure was to be investigated.

4 MATERIALS AND METHODS

4.1 Chemicals

Fluka (Neu Ulm, Germany)	Polyethylene glycol (PEG) [MW 5.000 – 7.000]
	D (-) Fructose
	Glucose oxidase from *Aspergillus niger*
	Sodium dodecyl sulfate (SDS)
	Potassium Chloride
Boehringer (Mannheim, Germany)	Glucose oxidase (GOD) from *Aspergillus niger*
Aldrich (Milwaukee, USA)	Poly-L-lysine [MW 30.000 - 70.000]
	Nafion®
Merck (Darmstadt, Germany)	D (+) Glucose anhydrate
	Hydrogen peroxide (30 %)
	L (+D) ascorbic acid
	Graphite
	Boric acid
	Sodium hydroxide
	Potassium dihydrogen phosphate
	Dipotassium hydrogen phosphate
	Hydrochloric acid
	N,N,N`,N`-Tetramethylethylenediamine (Temed)
	Tris(hydroxy methyl)-aminomethane (Tris)
	Calssium chlodide dihydrate
	Toluene
ISP (Guildford, UK)	Gafquat 755N
Sigma-Aldrich (Schnelldorf, Germany)	Didodecyldimethylammonium Bromide (DDAB)
	Poly(acrylic acid) (Average MW 450000 g/mol)
Sigma-Aldrich (Steinheim, Germany)	Cetyltrimethylammonium bromide (C_{16}TAB)
	Acrylamide
	2-acrylamido-2-methyl-1-propanesulfonic acid (AMPS)
	Vinylsulfonic acid sodium salt (VSA)
	Catechol
	3,4-Dihydroxyphenethylamine (Dopamine)

	3-Aminopropyltriethoxysilane (APTES)
	γ-butrolactone
	Bovine Serum Albumin (BSA)
	MES [(2-[N-Morpholino]ethanesulfonic acid]
	2,3-Dimethoxy-2-phenylacetophenone
	Gluteraldehyde
	Sucrose
	Ammonium hydroxide
	Xanthine (sodium salt)
LKB-Produkter AB (Bromma, Sweden)	γ-methacryloxy-propan-trimethoxy silane (MAPTES)
SERVA (New York, USA)	N,N`-methylenebisbis(acrylamide) (BIS)
BASF (Ludwigshafen, Germany)	Laromer® LR 9005 (aromatic polyurethane dispersion)
Marabuwerke GmbH & Co. KG (Tamm, Germany)	Siebdruck-Hilfsmittel (screen printed ink contains Hexane-1,6-diol diacrylate)
SERVA (Heidberg, Germany)	Dextran sulfate
GIBCO BRL, Life Technologies, INC. (Gaithersburg, USA)	Ammonium persulfate (APS)
Carl Roth (Karlsruhe, Germany)	Rotiphorose Gel 30 contain a mixture of 30% acrylamide and 0.8 % bisacrylamide
Millinckrodt Baker B.V. (Deventer, Holland)	Ethanol
Riedel-deHaen Ag, (Seelze, Germany)	Acetone

4.2 Solutions

4.2.1 Enzyme biosensors

Clark & Lubs buffer (pH 8.0) used as a carrier buffer in Flow Injection Analysis (FIA) was prepared by mixing 12.4 g boric acid and 14.9 g KCl in 2000 mL Milli-Q water. Then 500 mL of this solution was diluted by Milli-Q water to 1000 mL and with NaOH 0.1 M the pH was adjusted to 8.

A stock solution of 5 g/L glucose was prepared in Milli-Q water and other concentrations were prepared by the appropriate dilution. Enzyme and other additives were dissolved directly in the used buffer.

4.2.2 Food and cultivation samples

For the application of glucose biosensors, seven different juice samples (named with their carbohydrate content in the results and discussion part) were examined using 3 different degrees of dilution (1:10, 1:50, 1:100 for samples 2-7 and 1:200, 1:300 and 1:400 for sample 1) with Milli-Q water. Samples 5 and 6 were filtered through a 0.22-µm filter before dilution.

Cultivation samples from Escherichia coli (*E. coli)* were obtained from Dr. U. Rinas (BVT, Gesellschaft für Biotechnologische Forschung (GBF), Braunschweig, Germany), where the cultivation process was carried out in a complex medium starting with 20 g/L glucose. The samples were centrifuged for 20 min (at 14000 rpm), filtrated and diluted to 1:10 by Milli-Q water. All solutions were degassed just before their use in the FIA systems.

For comparison and evaluation of the resulted data from glucose detection obtained by FIA or from microchips capillary electrophoresis, a reference method from Yellow Springs Analyzer (YSI) (model 2000, Yellow Springs Instruments, Yellow Springs, OH, USA) was used.

4.2.3 Capillary electrophoresis and microfluidic chips

Phosphate buffer 10 mM (pH 7.3) was prepared by mixing suitable volumes of the stock solutions of 100 mM potassium dihydrogen phosphate and 100 mM dipotassium hydrogen phosphate. After preparation, the buffer was filtered by passing through a 0.22µm filter and stored at 4 ^{o}C. All solutions were degassed before use.

Tris-buffer was prepared by dissolving Tris(hydroxy methyl)-aminomethane in Milli-Q water and the pH 7.4 was adjusted by 1 M HCl.

Tris-Borate (100:150 mM) buffer pH 8 was prepared by dissolving the appropriate amounts from both Tris and boric acid in Milli-Q water.

10 mM borate buffer was prepared either by dissolving boric acid or sodium tetraborate in Milli-Q water and the pH was adjusted by 1 M NaOH.

MES buffer was prepared by dissolving the appropriate amounts in Mill-Q water and the pH was adjusted by 1 M NaOH.

100 mM stock solutions of each glucose, fructose, sucrose, ascorbic acid or H_2O_2 were prepared in phosphate buffer pH 7.3; other diluted solutions were prepared by the appropriate dilution.

CTAB, SDS and DDAB solutions were prepared in phosphate buffer 10 mM (pH 7.3). Another solution from DDAB was prepared by dissolving in 20 mM Tris-HCl + 20 mM $CaCl_2$.

Three different juice samples were tested for applications in microfluidic chips. These juice samples were diluted 1: 200 by phosphate buffer with CTAB (0.01 mM and 0.001 mM in case of glass and PMMA microchips, respectively). The diluted solutions were filtered through 0.22-µm filters.

4.3 Methods

4.3.1 Screen printed electrodes

The electrodes used in this study were screen-printed platinum (Pt) electrodes fabricated in the GBF (Braunschweig, Germany). As seen from Fig. 4.1 each electrode unit consisted of two working electrodes (with a half circle form with ~ 6.3 mm^2 surface area) and a reference electrode. Pt-electrodes were prepared by printing a Pt-paste (3804 Ferro, New York, USA) on high quality fired aluminum oxide ceramics (Ceramic Tech., Hoechst, Frankfurt, Germany) by using a conventional screen-printing machine (Microtronic II; Ekra, Kirchheim, Germany). The conducting paths and the isolating layer were applied by screen-printing of a dielectric paste (No. 5032, DuPont, USA). After each printing step the ceramic substrate was left at room temperature for 15 min, dried at the furnace at 120 ^oC for another 15 min followed by a firing process at 850 ^oC (Bruce 7354 –M, BTU Eng. Corp., UK) to get stable and fixed layers.

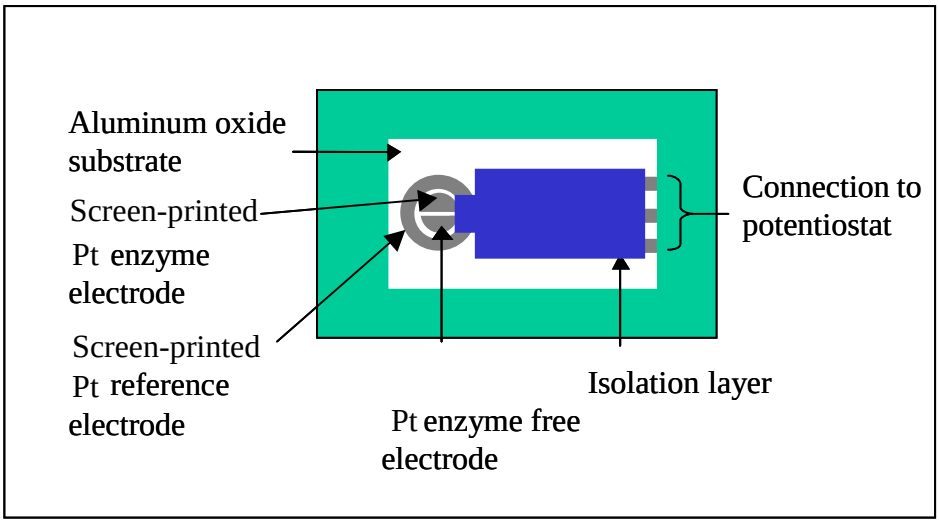


Fig. 4.1 The layout for screen-printed electrode.

4.3.2 Immobilization of GOD

On one of the two working electrodes (Fig. 4.1) glucose oxidase (GOD) was immobilized while the second electrode was screen-printed with a non-GOD membrane to monitor the influence of interferences of the components of the medium and to detect possible cross talk between the electrodes during standard measurements. GOD was immobilized by entrapment in a UV-polymerizable screen printable paste, based on polymethacrylate. The enzyme paste was prepared by mixing 35 mg of UV-paste with 10 µL GOD (60 mg/mL) and 10 µL Clark & Lubs buffer pH 8 (or 10 µL from an additive). The mixture was screen-printed by a manual screen-printing device (HAT 10, Fleischle, Brackenheim, Germany). After screen-printing, the enzyme pastes were fixed by irradiation with UV-light for ~ 3 s (UN 50011 Light system Type 14012, Aktiprint mini 12, Technigraf, Grävenwiesbach, Germany). The prepared electrodes were stored at 4 ^{o}C in a Clark & Lubs buffer.

4.3.3 Flow Injection Analysis (FIA)

FIA measurements were carried out using an automated TRACE TAS 2000 FIA device (TRACE Biotech AG, Braunschweig, Germany). This system (Fig. 4.2) contains two peristaltic pumps, one for the carrier buffer with a flow rate of 0.8 mL/min and the second for the sample solution. One injection valve with injection volume of 5 µL, one selection valve and the potentiostat completed the system. All units were automatically controlled by a PC using the TAS software (TRACE Biotech AG, Braunschweig, Germany).

Clark & Lubs buffer 0.1 M (pH 8) was used as a carrier buffer, flow rate 0.8 mL/min and 5 µL injection sample volume.

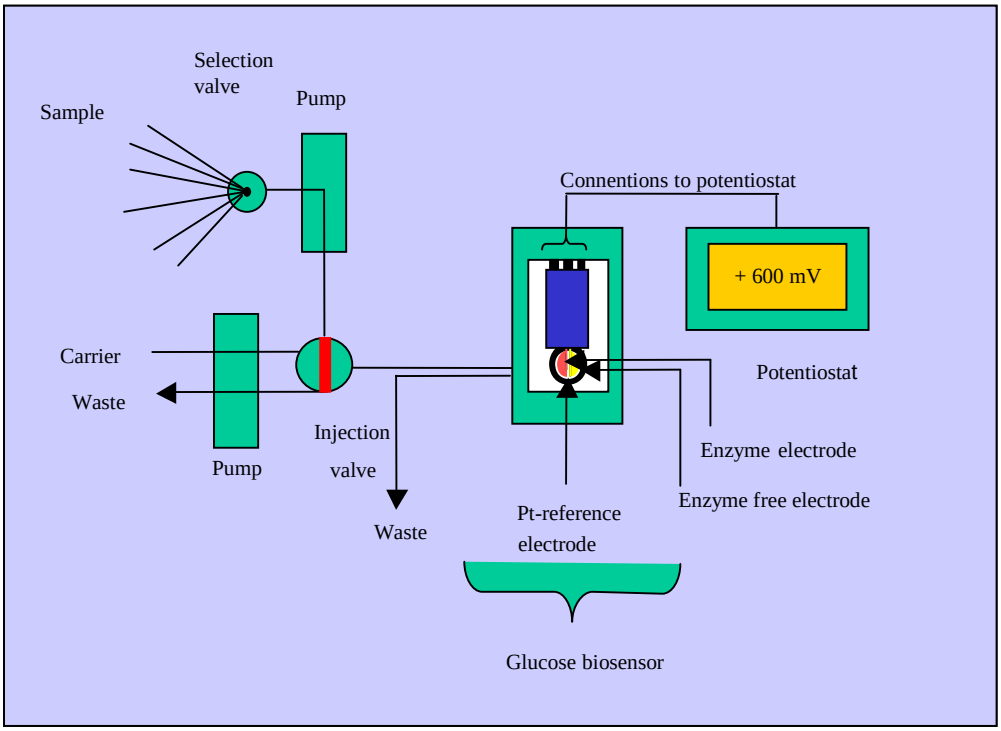


Fig. 4.2 Scheme for FIA system.

Glucose detection was based on the electrochemical oxidation of enzymatically produced hydrogen peroxide at the Pt-enzyme electrode. The measurements were carried out by application of + 600 mV (*vs.* Pt-screen-printed reference electrode) on the Pt-screen-printed enzyme electrode which was used as detector in the FIA-system. The electrode chip was placed in a wall-jet cell made from Plexiglas (GBF, Braunschweig, Germany) with a stainless steel capillary, used as inlet and also as auxiliary electrode. The electrodes were connected directly to the potentiostat unit of the FIA device and the data were recorded on a chart recorder BD 112 (Kipp & Zonen, Delft, The Netherlands).

4.3.4 Capillary electrophoresis set-up

Capillary electrophoresis measurements were carried out using a fused silica capillary with 50 µm i.d. obtained from Agilent Technologies (Böblingen, Germany) and fixed in the house made holder (Fig. 4.3) fabricated from Plexiglas (GBF, Braunschweig, Germany).

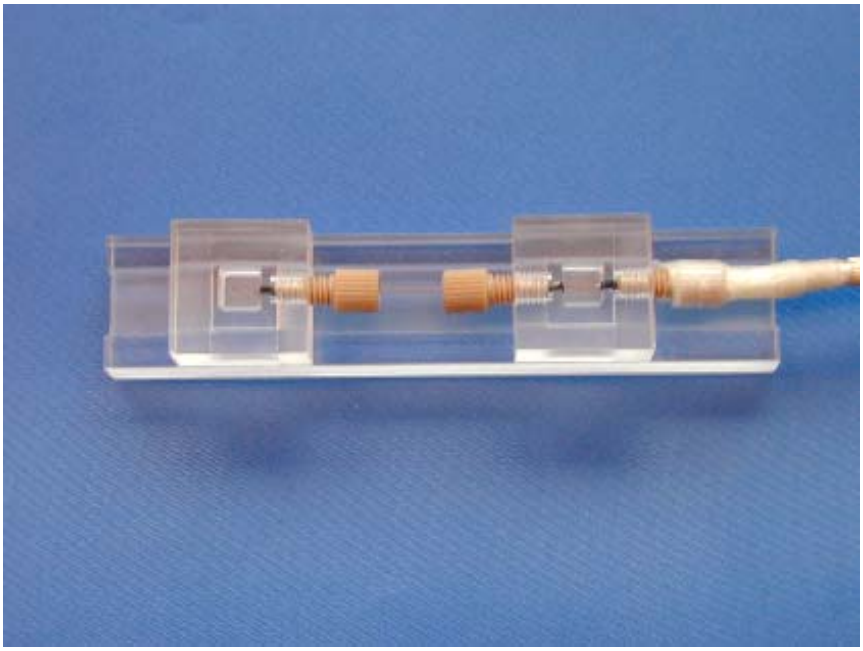


Fig. 4.3a The house made set-up for capillary electrophoresis.

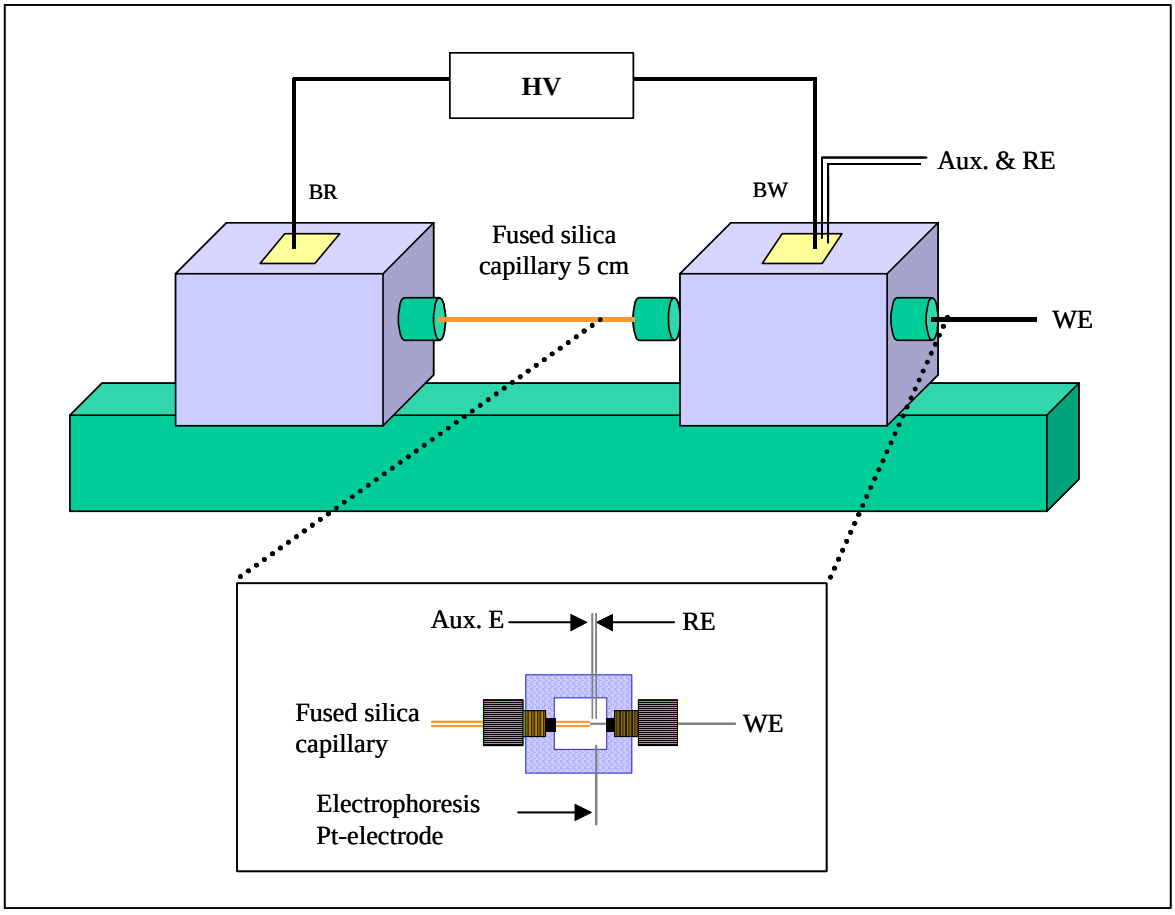


Fig. 4.3b The layout of the house made CE system.

4.3.5 Microfluidic chips

Three different kinds of microfluidic chips (glass, PMMA and ceramic) were used in this study. Glass microchips fabricated from borosilicate glass were obtained from Micralyne (Edmonton, Canada). Poly(methylmethacrylate) PMMA microchips were obtained from AMT (Application Centre for Microtechnology, Jena, Germany). Both types of microfluidic chips consisted of two crossed channels with 85 and 8 mm length for the long and the short channels, respectively; the intersection occurs 4 mm from the end of the long channel. The channels were 50 µm wide and 20 µm deep, the layout is shown in Fig. 4.4.

The third type of microchips were the ceramic microchips with integrated gold electrodes (Fig. 4.5). They were obtained from VIA electronic (Hermsdorf, Thüringen, Germany) and fabricated by Low Temperature Cofired Ceramic (LTCC) technology. The chips consisted of a 49 mm long separation channel and an 8 mm long injection channel.

1.0 mm Pt wires (Chempur, Karlsruhe, Germany) were inserted into the reservoirs and used as a connection from the high voltage power supply (FUG, HCN series, Rosenheim, Germany) with a +/- 12.5 kV high voltage power supply.

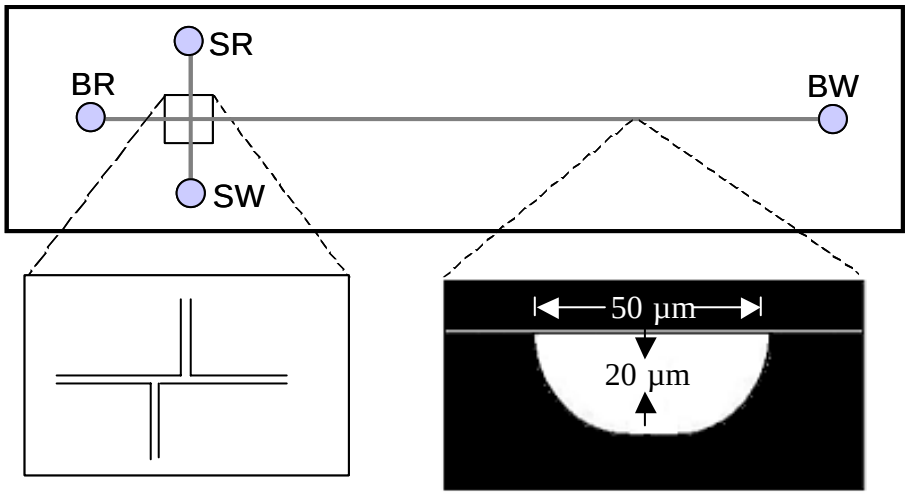


Fig. 4.4 The layout of microfluidic chips (glass and PMMA) with double T-intersection.

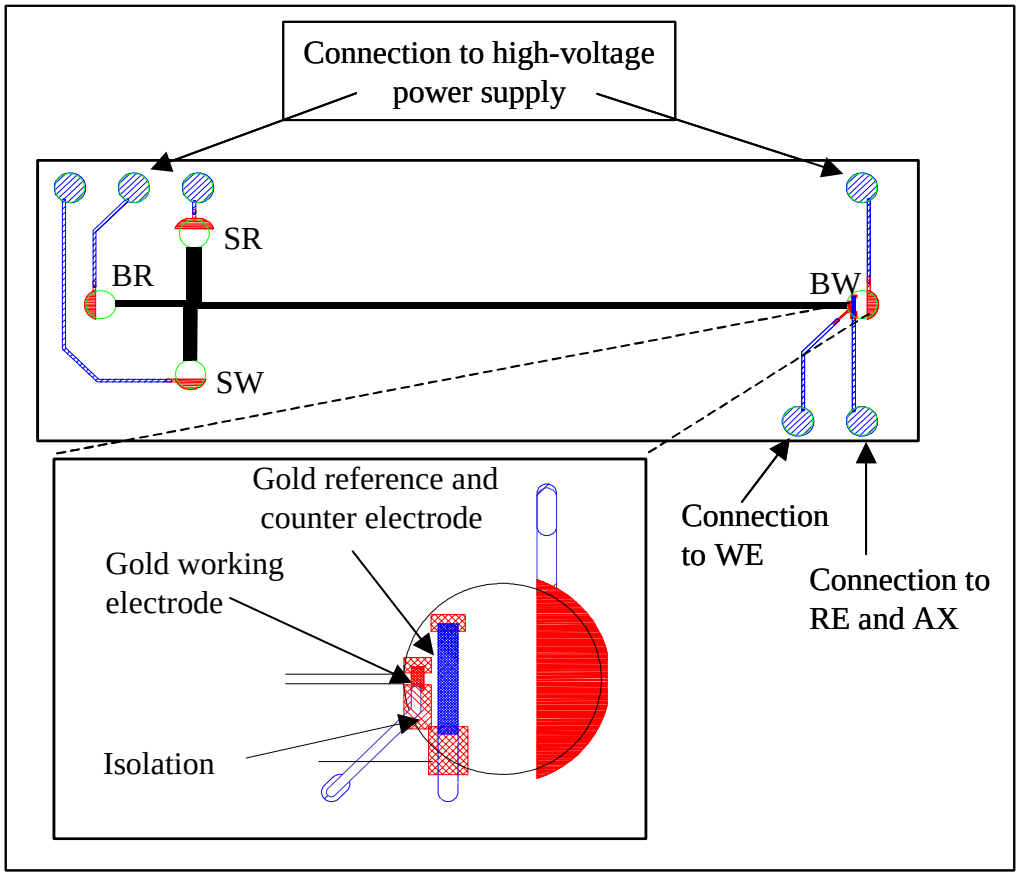


Fig. 4.5 The layout of ceramic microchips.

For the determination of glucose, the amperometric detection was performed using platinum wires (0.25 mm) as working, reference and counter electrodes connected to a Metrohm potentiostat (641 VA-Detector, Metrohm, Switzerland). The data were recorded on a chart recorder BD 112 (Kipp & Zonen, Delft, The Netherlands).

The separations in capillary electrophoresis and microfluidic chips were electrokinetically driven using a high-voltage power supply from Micralyne (Microfluidic Tool Kit Instrument, Micralyne Inc., Edmonton, Canada) with a high voltage from 0 to + 6 kV and controlled by a Labview program (version 5, National Instruments, München, Germany).

4.3.6 Electrophoresis procedures.

New fused silica capillaries and glass microchips were flushed by 1M HNO_3 and 1 M NaOH using a vacuum pump system (Crabtree et al. 2001), then rinsed by Milli-Q water. Before use, the channels were rinsed by NaOH 0.1 M for 15 min and Milli-Q water for 5 min, followed by filling with the running buffer for 15 min.

PMMA and ceramic microchips were rinsed only by Milli-Q water for 10 min and then by the running buffer for 15 min. The microchips were fixed in their respective holders fabricated from Plexiglas (GBF, Braunschweig, Germany).

4.3.6.1 Current-voltage measurements

The PMMA or ceramic chips were rinsed by Clark & Lubs buffer pH 8 (10 mM) for 15 min. High voltage electrodes were connected to the BR and BW reservoirs, different high voltages were applied to the BR, the BW grounded and the resulted currents were recorded after 3 min, when a stable current through the separation channel was obtained. The maximum high voltages which were applied to the different types of microchips are collected in Table 4.1.

Chip Material	Maximum high voltage applied (V)	High voltage range (V) for EOF	Effective capillary length (mm)
Glass	7250	1000 - 2000	85
PMMA	10000	600 – 2000	85
Ceramic	400	150 – 300	49

Table 4.1 The maximum high voltages which were applied on microchips and also the high voltages which were applied for EOF measurements.

4.3.6.2 EOF measurements

For the EOF measurements H_2O_2 was used as neutral marker. According to equation 2.19 the EOF is the transportation rate of a neutral compound and can be determined via its transportation time t through a capillary of an effective length L. The electroosmotic mobility is then calculated according to equation 2.24 by normalisation of the EOF with respect to the applied electric field strength.

The chips were rinsed by NaOH 0.1 M for 15 min, Milli Q water for 5 min followed by the running buffer (phosphate 10 mM pH 7.3, or phosphate in presence of surfactants) for 15 min. High voltage (HV) was applied (Table 4.1) to the BR and BW was grounded. Detection was based on amperometric detection of H_2O_2 with Pt wires as working, reference and counter electrodes placed in the BW as close to the end of the capillary as possible. A potential of + 700 mV was applied to the working electrode. After a constant base line was obtained the BR was filled by 1 mM H_2O_2 and the HV was again applied on the BR, while the BW was grounded and the short channel was left floating. Detector signals were recorded on the chart recorder BD 112 (Kipp & Zonen, Delft, The Netherlands) and the transportation time was measured using a stopwatch.

4.3.6.3 Dynamic coating for microchips

For dynamic coating of microchips, the channels were rinsed by the running buffer (phosphate pH 7.3) containing the chosen concentration of the used surfactant for 30 min

using a peristaltic pump. Between runs the microchips were rinsed by Milli-Q water for 5 min. Surfactant concentrations ranged from 0.001 mM to 2 mM.

4.3.6.4 Injection mode used in microfluidic chips

Injection of samples were accomplished by application of high voltage (for a certain seconds as mentioned in the results and discussion part) to the sample reservoir (SR) and grounding the buffer waste reservoir (BW), while the buffer reservoir (BR) and sample waste (SW) reservoirs left floating.

For separation the high voltage was applied to the BR and grounded the BW. 85% from the high voltage applied to the BR was applied to the SR and SW to prevent sample depletion (Fig.4.6).

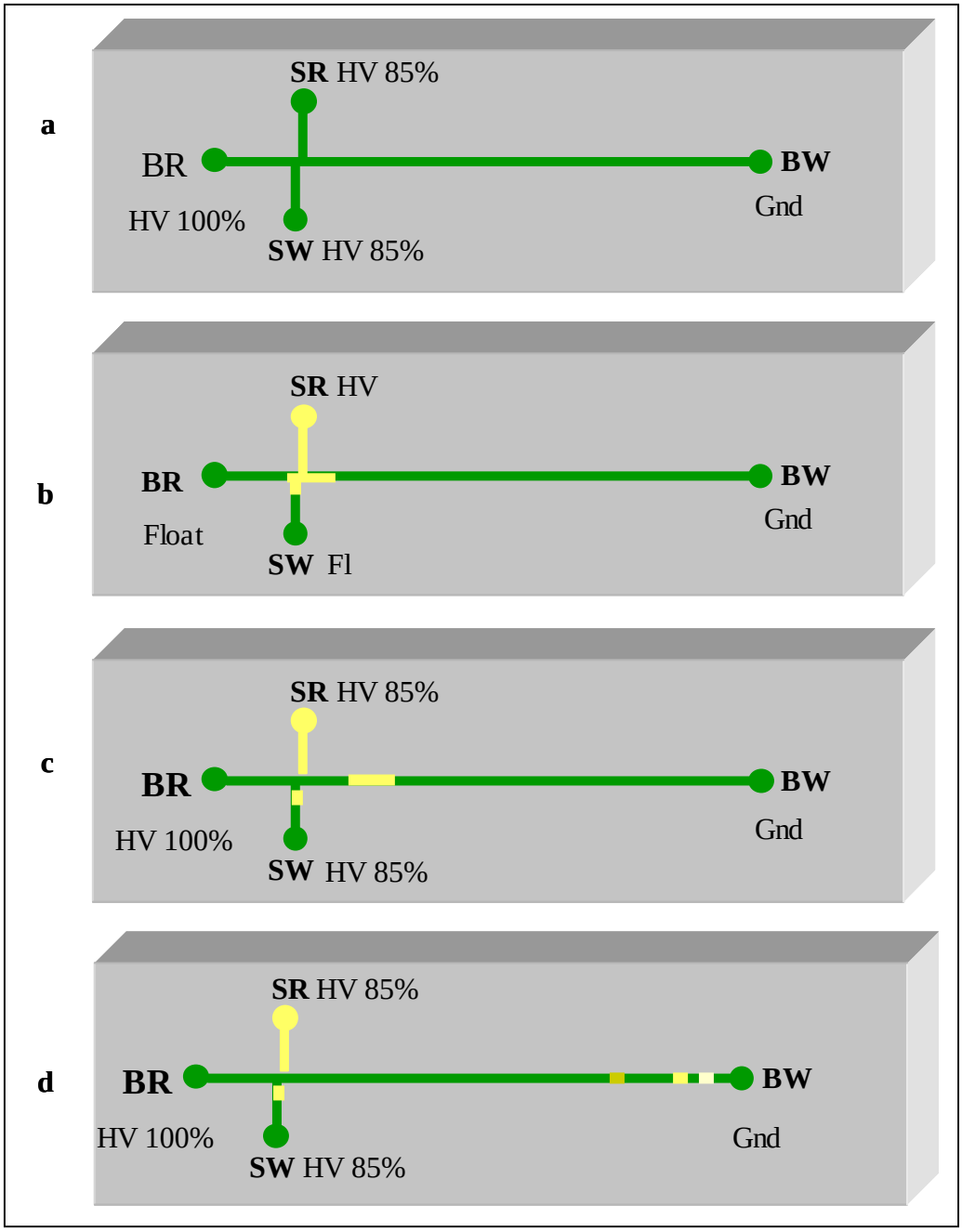


Fig. 4.6 Injection and separation modes were used in glass and PMMA microchips using the Micralyne system. (a) Conditioning the channels; (b) Injection; (c) Separation; (d) Detection.

4.3.6.5 Glucose determinations using soluble glucose oxidase (GOD)

The chip channels were rinsed with phosphate buffer (10 mM) pH 7.3 containing CTAB (0.01 and 0.001 mM in case of glass and PMMA microchips, respectively, for 30 min followed by filling with phosphate buffer containing CTAB and GOD. The microchips were fixed at a special holder fabricated from Plexiglas delivering reservoirs on top of the ends of the capillaries with volumes of approximately 100 µL. 50 µL from this solution were replaced by

either solutions of glucose, H_2O_2 or ascorbic acid, mixture of solutions from H_2O_2 and ascorbic acid or glucose and ascorbic acid. For sample injection the HV was applied for 10 s on the SR and the BW was grounded.

4.3.6.6 Immobilization of enzyme in fused silica capillaries and glass micochips using glutaraldehyde

The capillaries were treated by a mixture of 30% NH_3, 30% H_2O_2 and H_2O (1:1:5 by volume) at 100 ^oC for 5 min, followed by a mixture of 37% HCl, 30% H_2O_2 and H_2O (1:1:5 by volume) at 100 ^oC for 5 min. The capillaries were then rinsed by Milli-Q water, ethanol, and acetone, dried under a stream of air and then at 110 ^oC in an oven for 1 h.

The clean and dry capillary was washed with toluene and filled by a solution of APTES (15% in toluene) overnight for fused silica capillary and 10 min for glass microchips.

After removal of APTES from the capillary, the capillary was rinsed by toluene several times, acetone and then dried in the oven at 110 ^oC for 1 h.

The dried capillary was immersed in ethanol for 10 min followed by washing with phosphate buffer (pH 7) for 5 min.

The silanized capillary was filled with 2.5% glutaraldehyde in phosphate buffer for 1 h at room temperature, followed by rinsing with water to remove the unreacted traces from glutaraldehyde.

The capillary was filled by a mixture of glucose oxidase (60 mg/mL), BSA (10%) and glutaraldehyde 0.25% (1:1:1) for 1 h followed by rinsing with phosphate buffer. The immobilized capillary or glass microchip was stored in phosphate buffer at 4 ^oC.

4.3.6.7 Immobilization of glucose oxidase in glass capillary and glass microchip using polyacrylamide gel

4.3.6.7.1 Preparation of acrylamide solution

The acrylamide (AA)/bisacrylamide (BIS) solution was prepared by dissolving the suitable amounts from AA and BIS in Tris-boric acid buffer pH 8 (100 mM Tris and 150 mM boric acid) and degassed for 3 min. The total monomer concentration (%T) and the concentration of cross-linking agent (%C) were calculated by the following equations (Liu and Sweedler 1996):

$$\%T = \frac{\text{AA (g)} + \text{BIS (g)}}{\text{100 mL of solvent}}$$

$$\%C = \frac{\text{BIS (g)}}{\text{AA (g) + BIS (g)}}$$

A fixed amount of AMPS, VSA or poly(acrylic acid) (for generating the EOF) is defined as the molar ratio of the original acrylamide concentration i.e. a gel that solely consists of acrylamide and bisacrylamide. A certain amount of acrylamide had to be excluded and replaced by the AMPS, VSA or acrylamide.

To prepare an acrylamide mixture consisting of 5% T, 60% C, 3.9% acrylic acid and 3% PEG a 12.2 mg acrylamide, 30 mg BIS, 7.8 mg acrylic acid and 30 mg PEG were dissolved in 1 mL of Tris-boric buffer. To 250 µL of this mixture 1 µL from each 10% Temed and 1% APS freshly prepared were added and mixed very well. 2 µL of GOD 100 mg/mL were added to 18 µL of the last mixture and degassed for 3 min before filling the capillary.

4.3.6.7.2 Immobilization method

Immobilization of glucose oxidase in glass capillary was carried out according to the following method;

1. The capillary was rinsed by NaOH 1.0 M for 15 min followed by washing by Milli-Q water for 10 min using a simple vacuum system.
2. The capillary was washed by HCl solution 0.1 M for 20 min followed by Milli-Q water for another 10 min.
3. The capillary was incubated by 50% (v/v) γ-methacryloxy-propan-trimethoxy silane (MAPTES) in acetone for 30 min.
4. The capillary surface was washed by acetone, water and dried under dry air.
5. The polymerization mixture was filled into the capillary in the presence of the initiator.

For the immobilization of the enzyme in glass microchips the following procedure was used:

1. The buffer, buffer waste and sample reservoirs were filled by NaOH 0.1 M. Solutions were sucked through the chips by the application of vacuum to the sample waste reservoir. All channels were rinsed with NaOH for 30 min, Milli-Q water for 10 min, HCl 0.1 M for 20 min, and finally with Milli-Q water for another 10 min.
2. Buffer and buffer waste reservoirs were filled by Tris-boric acid buffer (100 mM Tris and 150 mM boric acid) pH 8, while the sample reservoir was filled by MATPES 50% (v/v) in acetone. Thus by sucking via the sample waste reservoir the injection channel was filled by MAPTES for 10 min.

3 Channels were flushed by acetone, Milli-Q water and dried under a stream of air.

4 Buffer and buffer waste reservoirs were filled by Tris-boric acid buffer and the sample buffer reservoir was filled by the acrylamide gel mixture and kept in it.

5 Polymerization was completed in Tris-boric acid buffer at room temperature overnight and microchips were stored in the same buffer at 4 ^{o}C.

4.3.6.7.3 Measurement of GOD activity in presence of different additives

1 µL from GOD 100 mg/mL was added to 1 mL phosphate buffer pH 7.3 (10 mM) in the presence of 0.025%, 0.1% and 1% additives VSA or AMPS in a small tube. Pt- wires (0.25 mm) were used as working, reference and auxiliary electrodes, + 700 mV potential was applied to the working electrode. After a stable base line was obtained, 1 mM glucose was added and the resulted signal was recorded.

5 RESULTS AND DISCUSSION

5.1 Determination of glucose by enzyme electrodes

Glucose oxidase was immobilized on screen-printed Pt-electrodes by entrapment within a screen-printable paste polymerized by irradiation with UV-light (Rohm et al. 1995). The composition of the immobilization matrix was to be optimised with respect to optimal sensitivity, stability and selectivity of the enzyme electrode. Prior to these investigations other experimental conditions were evaluated.

5.1.1 Influence of the applied potential

The influence of the applied electrode potential on the oxidation of hydrogen peroxide was studied in the potential range from + 200 to + 900 mV (*vs*. screen printed Pt-reference electrode). The enzyme layer consisted of GOD 100 mg/mL, BSA 20% and poly-L-lysine 20% according to the composition developed by Schumacher et al. 1999. The effect of the applied electrode potential was examined by injection of 5 g/L glucose solution. By increasing the electrode potential, glucose signals increased until a stable value was reached at + 800 mV (Fig. 5.1). For further investigations an electrode potential of + 600 mV was selected, as higher electrode potentials increased the possibility for interferences from other electroactive compounds.

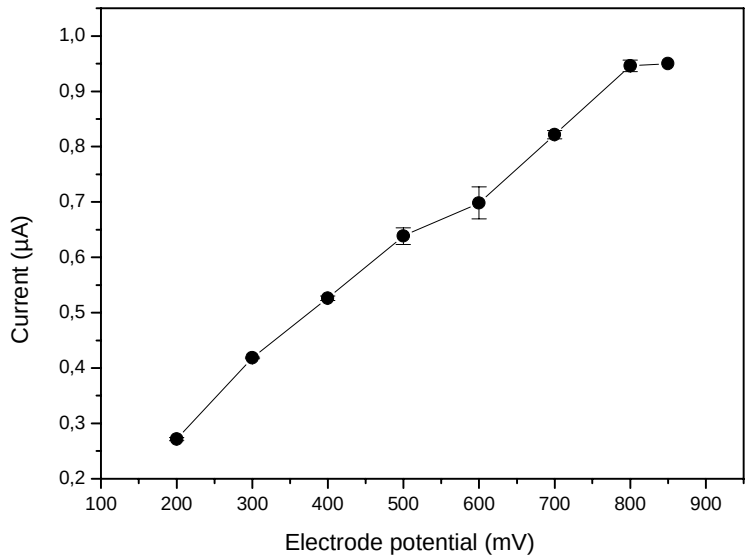


Fig. 5.1 The effect of electrode potential on the response of 5 g/L glucose.

5.1.2 Influence of pH and carrier buffer

The pH of the carrier buffer affects both enzymatic and electrochemical reactions. To investigate the effect of the pH of the carrier buffer on glucose signals, the pH range from 6 to 10 using Clark & Lubs buffer were examined. Glucose signals increased by increasing the pH of the carrier buffer from 6 to 7.5, then glucose signals slightly decreased at pH 8, while higher pHs, the signals decreased sharply (Fig. 5.2a).

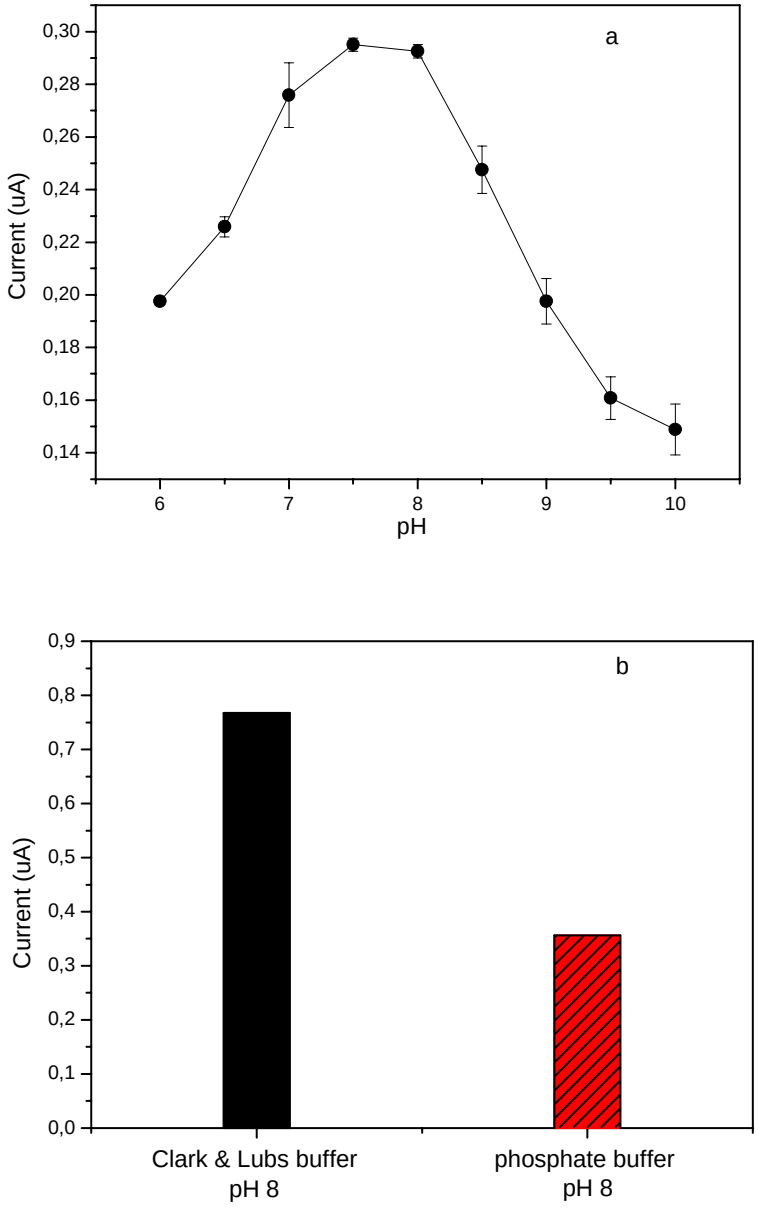


Fig. 5.2 The effect of (a) pH (b) kind of carrier buffer on the signal of 5 g/L glucose, conditions as in the experimental section.

Also the influence of the kind of the carrier buffer was studied using two different buffers pH 8. As seen from Fig. 5.2b glucose gave a higher signal when the Clark & Lubs buffer pH 8

was used (about 3 times the value in case of phosphate buffer). So for further investigations Clark & Lubs buffer (pH 8) was used.

5.1.3 Influence of enzyme concentration

The effect of different concentrations of GOD (20-100 mg/mL) on the glucose signal was investigated. By increasing the GOD concentration from 20 to 60 mg/mL the current increased, while at higher concentrations the obtained signals decreased (Fig. 5.3).

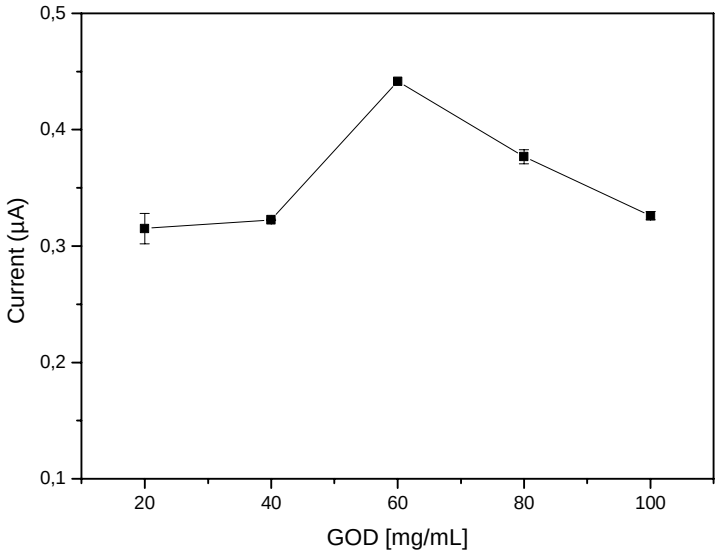


Fig. 5.3 The influence of GOD concentration on the response of 5 g/L glucose.

The immobilization of enzyme may have a significant effect on the stability of the enzyme. This was investigated during continuous work for 10 h with repeating the experimental program after 30 min. If GOD-solutions with 20, 40 and 60 mg/mL GOD were used, the enzyme electrodes showed almost no change of signals, whereas glucose signals increased after 4 h continuous work, if the GOD-concentration was increased to 80 and 100 mg/mL as shown in Fig. 5.4.

The stability of the glucose electrodes during long-term storage was investigated by storing electrodes at 4 ^{o}C in Clark & Lubs buffer pH 8.0 for 20 days. The stored electrodes showed a good stability during the studied storage period (Fig. 5.5).

For further studies a GOD-concentration of 60 mg/mL was selected not only due to the higher signal obtained, but also for the higher stability during continuous work.

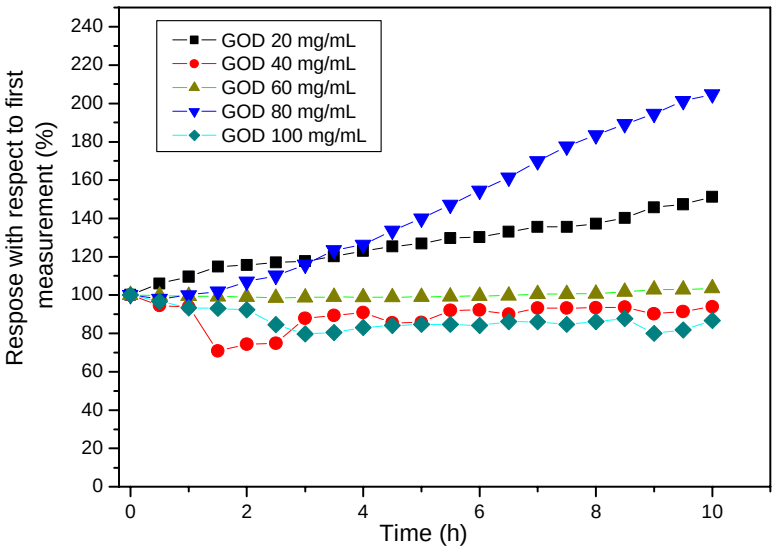


Fig 5.4 Stability curves for different enzyme electrodes using different GOD concentrations using 5 g/L glucose and samples were injected each 30 min.

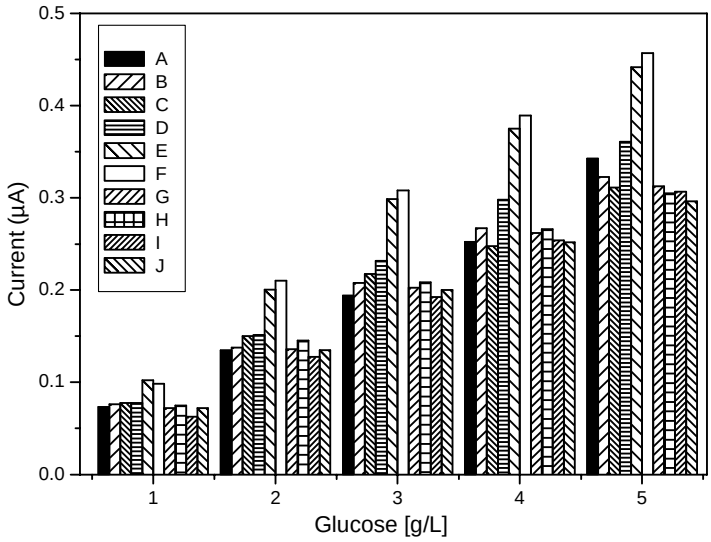


Fig. 5.5 Signals for for different glucose concentrations obtained with different screen printed GOD electrodes after preparation and after 20 days storage in Clark & Lubs buffer pH 8. (A) GOD 20 mg/mL; (B) GOD 20 mg/mL after 20 days; (C) GOD 40 mg/mL; (D) GOD 40 mg/mL after 20 days; (E) GOD 60 mg/mL; (F) GOD 60 mg/mL after 20 days; (G) GOD 80 mg/mL; (H) GOD 80 mg/mL after 20 days; (I) GOD 100 mg/mL; (J) GOD 100 mg/mL after 20 days.

5.1.4 **The selectivity of GOD screen-printed electrodes**

5.1.4.1 Response of ascorbic acid in presence of GOD

To study the effect of interferences on the selectivity of enzyme biosensors, ascorbic acid was used as a model compound. Two concentrations of ascorbic acid were applied to the different glucose electrodes under the same conditions as chosen for glucose analysis (Fig. 5.6). The resulting oxidation currents decreased by increasing the GOD concentration and were even higher than glucose signals (compare sigals in Fig. 5.3 and 5.6). Ascorbic acid gave oxidation signals also at the working electrode covered only with a screen-printed, UV-polymerized paste without enzyme. But these signals were smaller than those obtained at the GOD electrode.

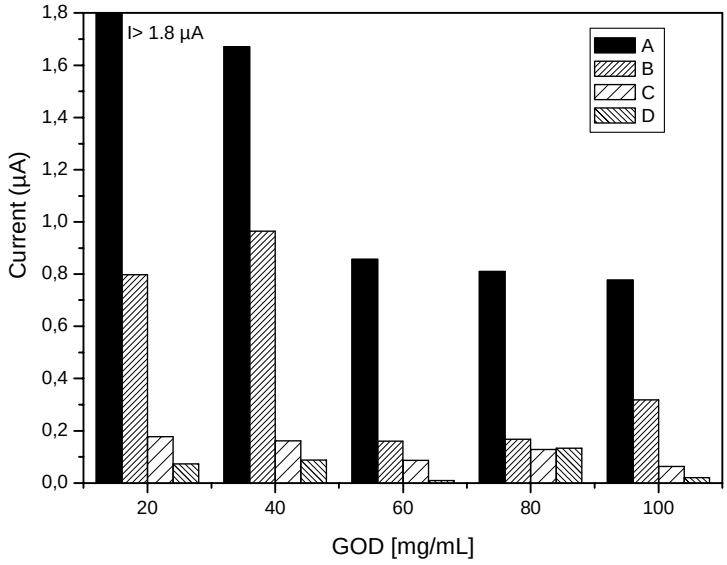


Fig. 5.6 Response of ascorbic acid using different GOD electrodes. (A) 1 g/L ascorbic acid (enzyme electrode); (B) 1 g/L ascorbic acid (enzyme free electrode); (C) 0.1 g/L ascorbic acid (enzyme electrode); (D) 0.1 g/L ascorbic acid (enzyme free electrode).

5.1.4.2 Response of xanthine in presence of GOD

Xanthine is another example for compounds, which may interfere with glucose determination at an enzyme electrode. Its oxidation was studied using the two concentrations 0.1 and 1.0 g/L. Xanthine showed higher signals at enzyme electrodes than at electrodes without enzyme as shown in Fig. 5.7. By increasing the enzyme concentrations, the xanthine signals decreased Obviously, the enzyme membrane had a high permeability for both ascorbic acid and xanthine and this permeability of the enzyme layer decreased by increasing the GOD concentration.

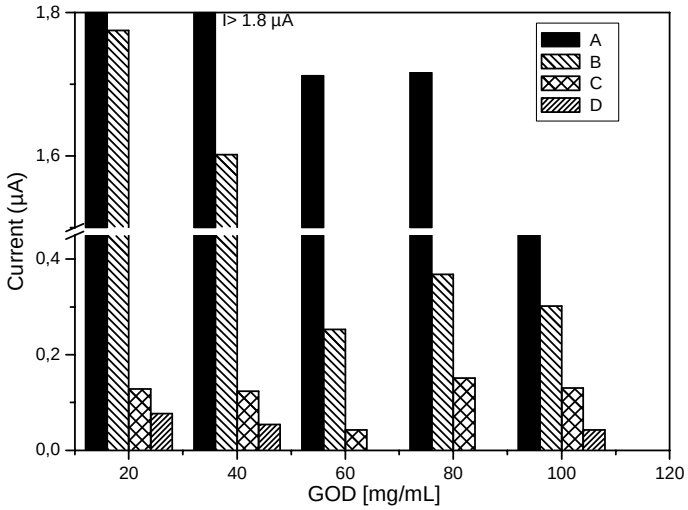


Fig. 5.7 Response of xanthine using different GOD electrodes. (A) 1 g/L xanthine (enzyme electrode); (B) 1 g/L xanthine (enzyme free electrode); (C) 0.1 g/L xanthine (enzyme electrode); (D) 0.1 g/L xanthine (enzyme free electrode).

5.1.4.3 Influence of 0.1 g/L ascorbic acid on glucose signals

The effect of 0.1 g/L ascorbic acid on the glucose signals was examined using GOD concentrations from 10 to 100 mg/mL. The enzyme working electrodes were prepared by mixing the desired GOD concentrations with the screen-printable paste and exposing to the UV-light, while the second working electrodes without enzyme were prepared by mixing the paste with an equal amount of the inert protein BSA. The difference between the two working electrodes gives the net value for the glucose signal. Different glucose concentrations were tested and the resultant signals were recorded in the absence and presence of 0.1 g/L ascorbic acid (Fig. 5.8). As expected from Fig. 5.6 ascorbic acid interferes with glucose and led to higher signals in the presence of ascorbic acid than for glucose only. Due to the higher permeability of the enzyme layer than of the BSA-layer, the effect of ascorbic acid oxidation could not be eliminated by the difference signals between both electrodes.

Thus, the influences of some additives were studied to reduce or eliminate the effect of ascorbic acid interferences with glucose. These additives included Gafquat 755N, poly-L-lysine, BSA, SDS, PEG, Nafion and graphite.

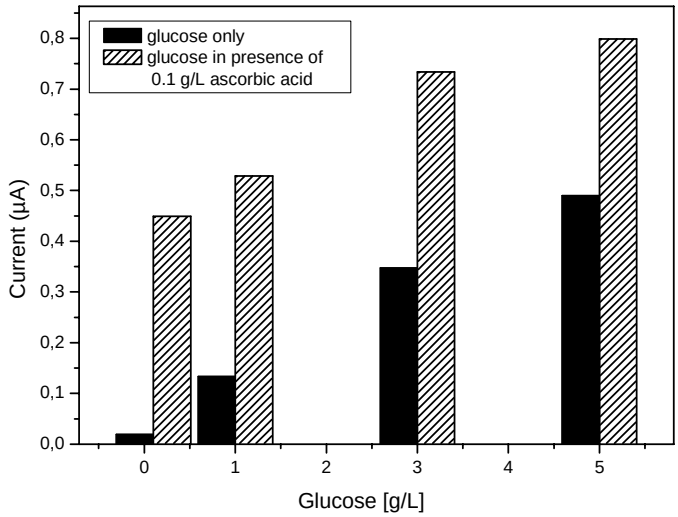


Fig. 5.8 Glucose signals obtained in the absence and the presence of 0.1 g/L ascorbic acid. 60 mg/mL GOD were used for immobilization.

5.1.5 Influence of some additives on the sensitivity and stability of screen-printed GOD electrode

Some additives showed a marked effect on the sensitivity when added to the enzyme electrode, for example the addition of poly-L-lysine, lactitol/dextran, lactitol/BSA/dextran, and BSA/poly-L-lysine to screen-printed xanthine electrodes increased the initial response for xanthine (Schumacher et al. 1999).

To investigate the effect of additives on the sensitivity, stability and selectivity of the screen-printed GOD (60 mg/mL) electrodes several types of additives were studied using different concentrations (from 0.05% to 20%).

5.1.5.1 Gafquat 755N

Schumacher et al. (1999) reported that the addition of Gafquat 755N or DEAE-dextran to dissolved horseradish peroxidase increased its sensitivity approximately 2.5-fold, but the activity decreased rapidly during the first 3 days and was decreased by about more or less 30% after 14 days.

Gafquat 755N is a quaternary copolymer formed from a combination of vinyl pyrrolidone and dimethylaminoethyl methacrylate with a pK_a of approximately 14 (Gibson et al. 1993). It is ionized at pH values below 14 and always positively charged in solution (Fig. 5.9). To investigate the effect of Gafquat 755N on the screen-printed glucose electrode, Gafquat 755N was mixed in different concentrations with GOD and the UV paste before immobilization. This lead to an increase of the sensitivity of the glucose electrode (Fig. 5.10).

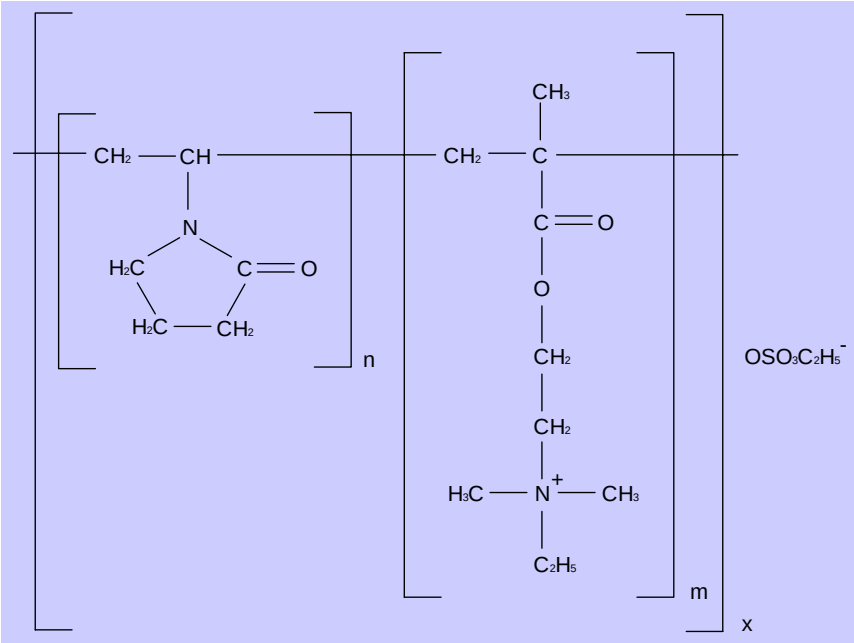


Fig. 5.9 Molecular structure for Gafquat 755N.

By addition of 1% Gafquat 755N to the enzyme membrane glucose signals decreased while by addition of 5% and higher concentrations the obtained signals increased with a maximum value at 10% Gafquat (Fig. 5.10). Higher concentrations from Gafquat 755N lead to a small decrease in the sensitivity of the enzyme electrode. Probably the small concentrations of Gafquat 755N (5 to 10%) increased the permeability of the membrane while higher concentrations increased the layer density and reduced the permeability of glucose through the membrane.

It was reported that addition of polyelectrolyte mixtures to soluble or immobilized enzymes produced an approximately equivalent stability effect due to the formation of protein-polyelectrolyte complexes (Gibson el al. 1996). Thus, the effect of the addition of Gafquat 755N on the stability of the glucose biosensors was investigated. As shown in Fig. 5.11, all enzyme electrodes with Gafquat 755N besides the enzyme electrode with 1% Gafquat 755N showed a slight decrease in their activity during the first 3 h. The enzyme electrode with 5% Gafquat 755N showed the highest decrease in the activity while, the smallest decrease was observed with the electrode with 20% Gafquat.

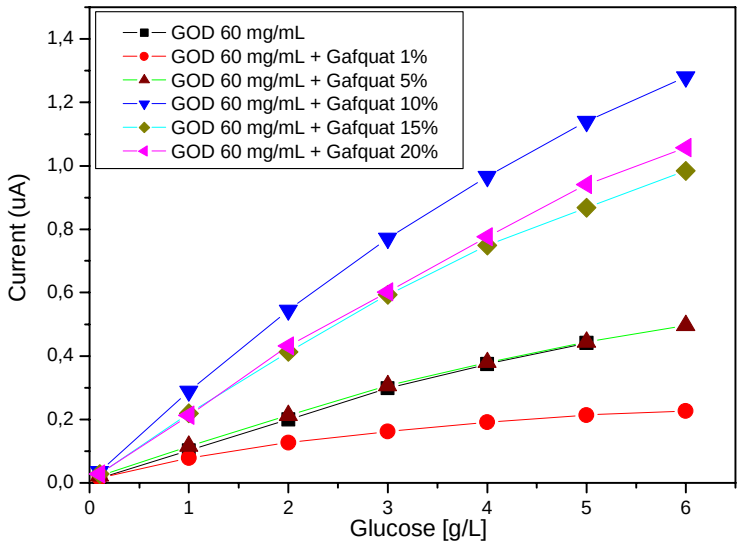


Fig. 5.10 Effect of different concentrations of Gafquat 755N on the sensitivity of GOD.

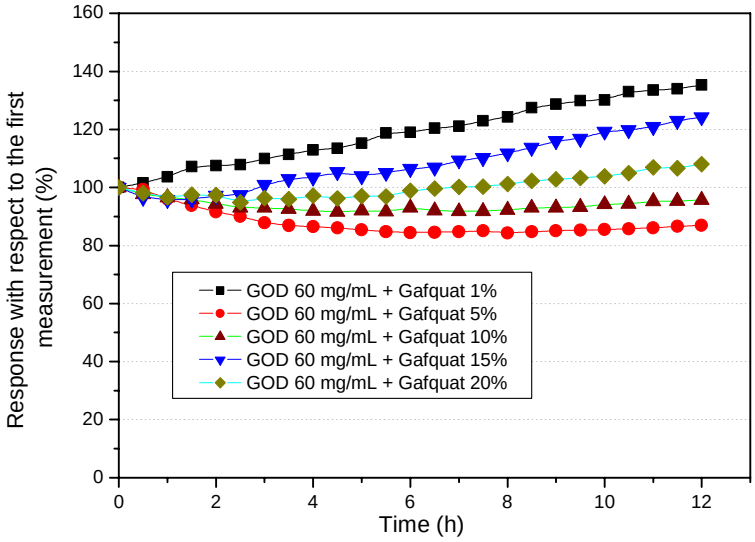


Fig. 5.11 Stability curves for screen-printed enzyme electrodes using different Gafquat concentrations. Injection of a 5 g/L glucose solution each 30 min.

As glucose biosensors without additives showed no loss in the activity, the addition of Gafquat 755N led to no improvement, the addition of 5% and 10% even decreased the stabilities (Fig. 5.4 and 5.12).

The response to ascorbic acid was examined at GOD electrodes with different Gafquat 755N concentrations (from 1 to 20%) while the second working electrode was prepared with Gafquat 755N without GOD. The glucose electrode with 1% Gafquat 755N showed a small response to ascorbic acid. Increasing the Gafquat 755N content in the enzyme layer increased

the permeability of the membrane for ascorbic acid. Fig. 5.12 shows the signals for 0.1 and 1.0 g/L ascorbic acid. With 1 g/L ascorbic acid high signals were obtained with all Gafquat concentrations. The same behaviour was obtained for the enzyme free electrode. Only the addition of 5% Gafquat led to lower signal. Using the lower ascorbic acid concentration (0.1 g/L) ascorbic acid showed signals at both the enzyme and the enzyme free electrodes and the signals at the enzyme electrode were higher than at the enzyme free electrodes except in the case of 5% Gafquat (Fig. 5.13). This may be due to the increase of Gafquat concentrations increased the permeability of the enzyme layer to ascorbic acid.

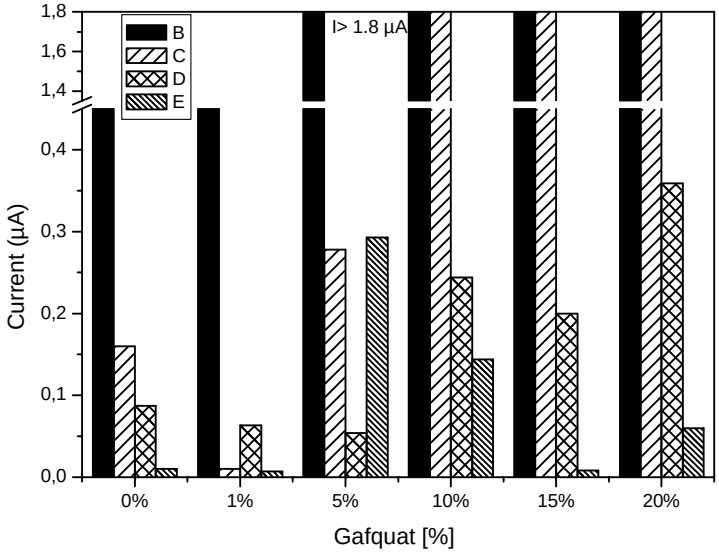


Fig. 5.12 Response of ascorbic acid at enzyme electrodes with different Gafquat 755N concentrations. (A) 1 g/L ascorbic acid at enzyme electrode, (B) 1 g/L ascorbic acid at enzyme free electrode, (C) 0.1 g/L ascorbic acid at enzyme electrode, (D) 0.1 g/L ascorbic acid at enzyme free electrode.

In a comparable procedure the permeability of the enzyme membrane for xanthine was studied. Xanthine showed a different behaviour than ascorbic acid, the permeability of xanthine at the enzyme free electrode was higher than at the enzyme electrode with Gafquat. At 5% Gafquat the permeability of the free enzyme membrane for xanthine is much higher than in enzyme electrode. At higher Gafquat 755N concentrations the permeability for xanthine was very high in both two working electrodes (Fig. 5.13).

For the electrode containing 1% Gafquat in the enzyme layer 1:1 and 10:1 mixtures of glucose and ascorbic acid were examined (Fig. 5.14). Significant signal increases due to the presence of ascorbic acid were obtained, even when the difference between the signal from the enzyme

electrode and from the enzyme-free electrode was taken. This indicates again the different permeability of both layers and poor selectivity of these electrodes.

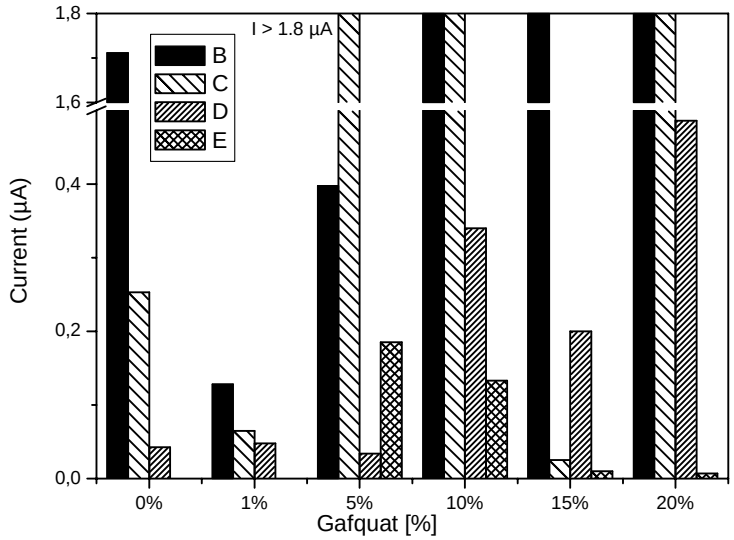


Fig. 5.13 Response of xanthine at enzyme electrodes with different Gafquat 755N concentrations. (A) 1 g/L xanthine at enzyme electrode, (B) 1 g/L xanthine at enzyme free electrode, (C) 0.1 g/L xanthine at enzyme electrode, (D) 0.1 g/L xanthine at enzyme free electrode.

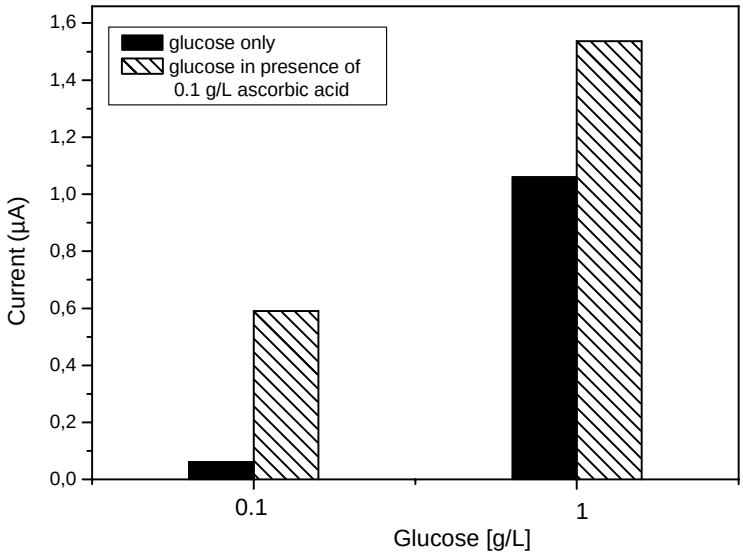
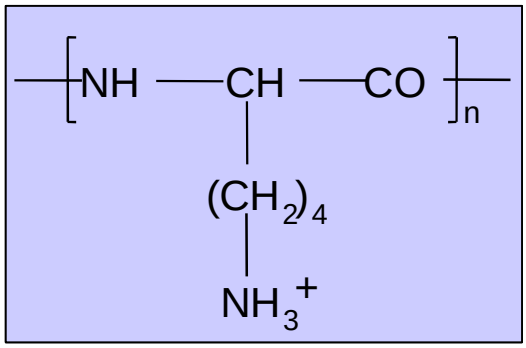


Fig. 5.14 Effect of 0.1 g/L ascorbic acid on the response of 0.1 and 1 g/L glucose using an enzyme layer with 1% Gafquat.

5.1.5.2 Poly-L-lysine

Poly-L-lysine is a positively charged polypeptide (Fig. 5.15), which leads to an enhancement of glucose signal when added to GOD during the preparation of the glucose biosensor. The addition of 1% of poly-L-lysine increased the signal of glucose by more than 3-fold compared to signals obtained with an electrode without poly-L-lysine (Fig. 5.16).



ig. 5.15 Chemical structure of poly-L-lysine.

Different poly-L-lysine concentrations were added to the GOD electrode and their influence on the calibration curve was studied. The addition of 1% poly-L-lysine led to the highest glucose signals, while 5% and 10% solutions showed just a slightly positive effect Fig. 5.16.

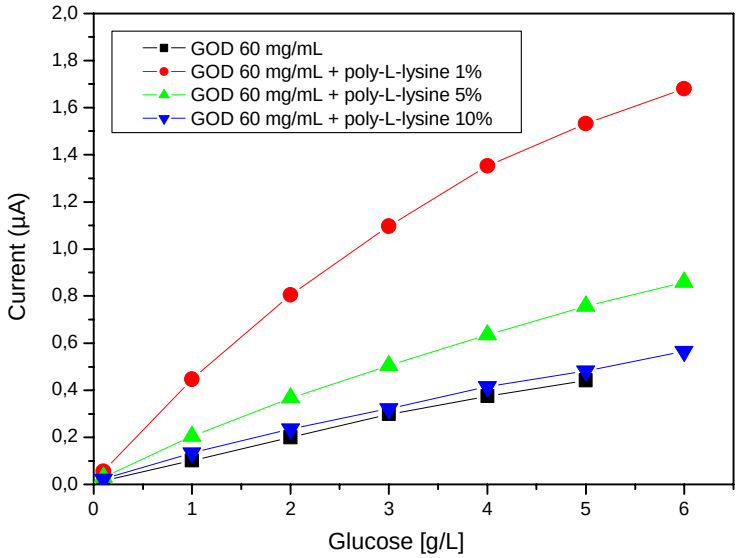


Fig. 5.16 Effect of different concentrations from poly-L-lysine on the signals of different glucose concentrations.

The permeabilities of these membranes for ascorbic acid and xanthine were also investigated. The presence of poly-L-lysine increased the permeabilities of the membranes for ascorbic acid (Fig. 5.17) and xanthine (5.18) irrespective of the poly-L-lysine concentrations. Due to

the increased permeability of membranes containing poly-L-lysine for ascorbic acid, the presence of 0.1 g/L ascorbic acid in solutions with 0.1 and 1 g/L glucose increased the response of the enzyme electrode significantly (Fig. 5.19). Thus, these enzyme electrodes showed a poor selectivity for glucose. This may be due to the positive charge on the enzyme membrane from the polycationic poly-L-lysine, which increased the interaction with the negatively charged ascorbic acid.

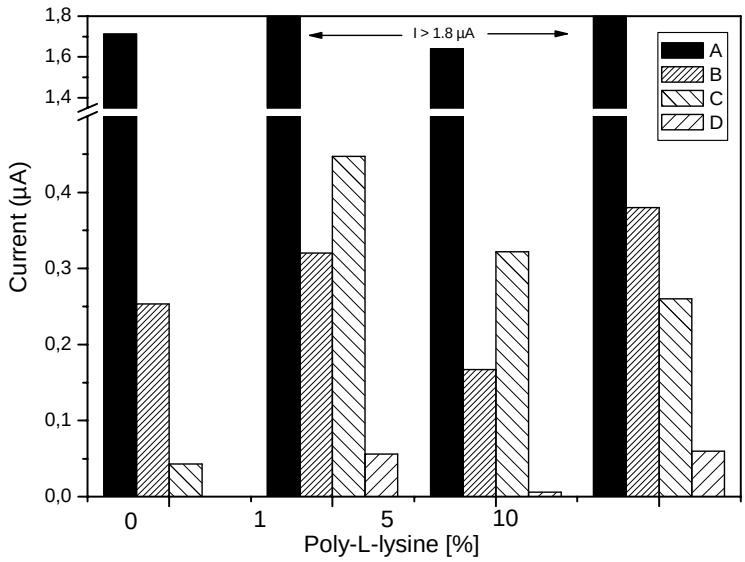


Fig. 5.17 Signals for two different ascorbic acid concentrations at electrodes with various poly-L-lysine concentrations. (A) 1 g/L ascorbic acid at enzyme electrode, (B) 1 g/L ascorbic acid at enzyme free electrode, (C) 0.1 g/L ascorbic acid at enzyme electrode, (D) 0.1 g/L ascorbic acid at enzyme-free electrode.

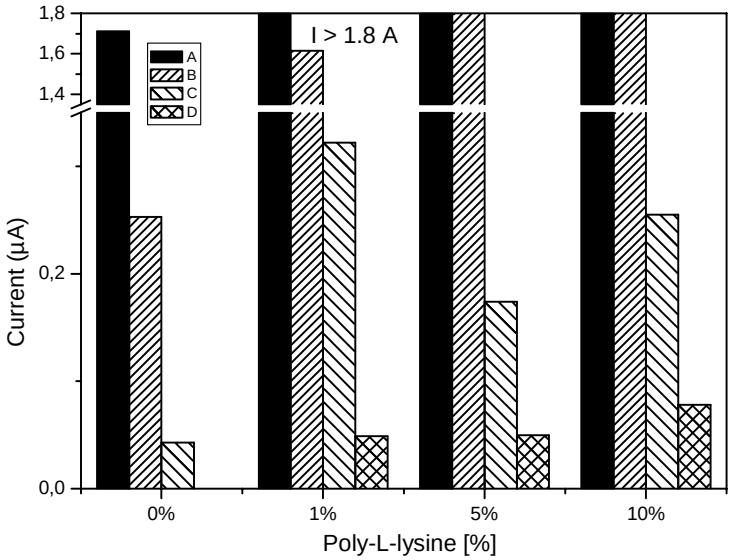


Fig. 5.18 Signals for two different xanthine concentrations at electrodes with various poly-L-lysine concentrations. (A) 1 g/L xanthine at enzyme electrode, (B) 1.g/L xanthine at enzyme free electrode, (C) 0.1 g/L xanthine at enzyme electrode, (D) 0.1 g/L xanthine at enzyme free electrode.

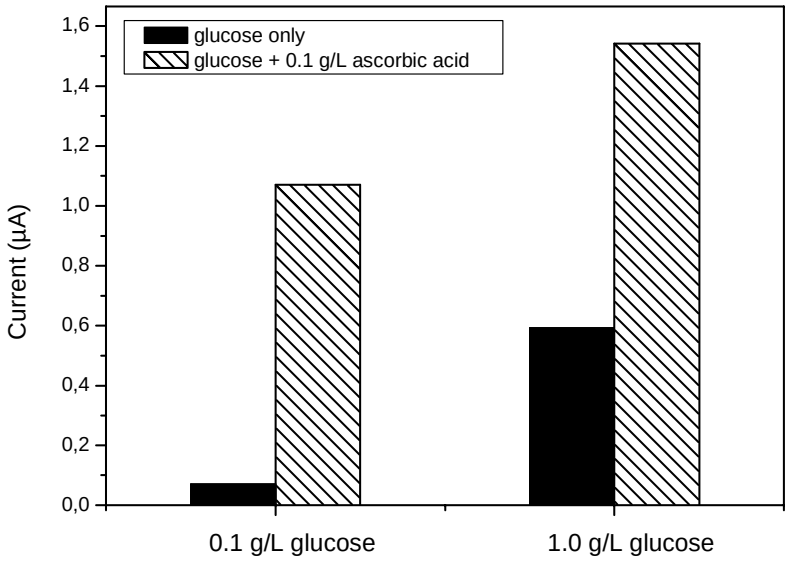


Fig. 5.19 The effect of the presence of ascorbic acid on the response of glucose, using enzyme electrode prepared 1% poly-L-lysine.

5.1.5.3 BSA

BSA was added during the immobilization of enzymes to increase the stability of the prepared biosensors. Addition of BSA from 1% to 20% had no effect on the sensitivity of glucose signals, while the addition of 25 and 30% increased glucose signals ~ 2 and 3 fold, respectively (Fig. 5.20).

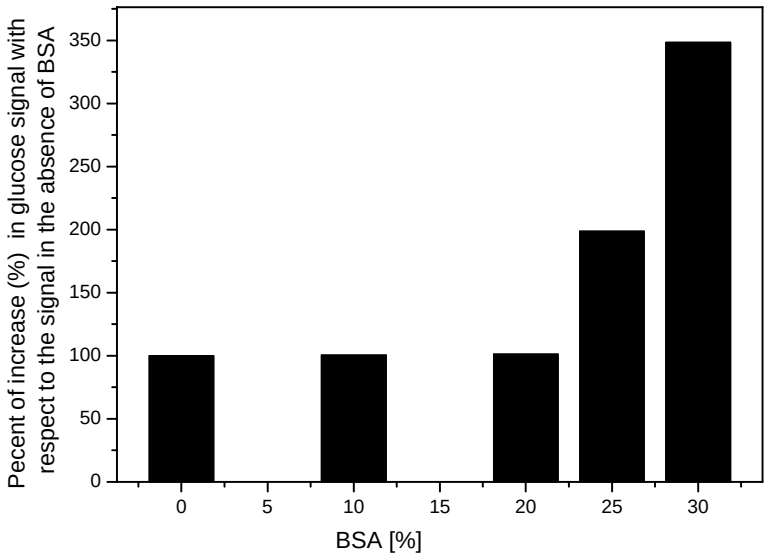


Fig. 5.20 Effect of the BSA addition on the signal of 5 g/L glucose.

The selectivity of glucose oxidase membrane containing 20% BSA was investigated. In a mixture of glucose and ascorbic acid the difference between the two working electrodes was increased by the presence of 0.1 g/L ascorbic acid (Fig. 5.21). Thus, also the presence of BSA could not eliminate the interferences from ascorbic acid.

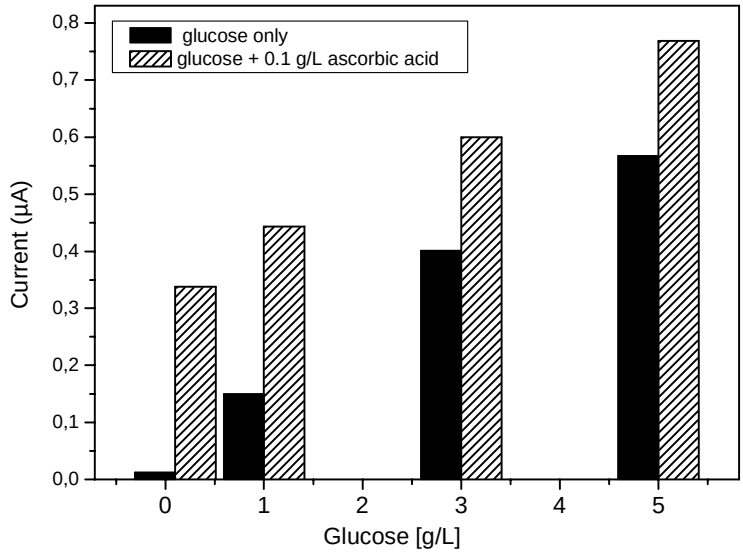


Fig. 5.21 The effect of presence of ascorbic acid on the response of glucose using GOD 60 mg/mL with 20% BSA.

5.1.5.4 SDS

The influence of the negatively charged surfactant SDS on the response of a glucose electrode containing already poly-L-lysine in the enzyme layer was investigated. SDS was added with

1% poly-L-lysine to the enzyme solution mixing with the UV paste before immobilization. With comparison to the signal obtained in glucose electrode in presence of 1% poly-L-lysine only and in presence of SDS, the addition of 10% SDS, glucose signal decreased by ~ 42%, while glucose in presence of 0.1 g/L ascorbic acid decreased by ~ 71% (Fig. 5.22). Addition of 20% SDS decreased the signals of 1 g/L glucose by 73%, and by ~ 92% in case of 1 g/L glucose in the presence of 0.1 ascorbic acid (Fig. 5.22). So addition of SDS to the enzyme electrode reduced partially the interference due to the presence of ascorbic acid. Decreasing glucose signal by increasing SDS concentration from 10 to 20% may be due to an effect of SDS on the activity of the enzyme. The addition of another layer containing the polymers on top of the enzyme membrane reduced both glucose and ascorbic acid signals without any effect on the difference signal. (Fig. 5.22).

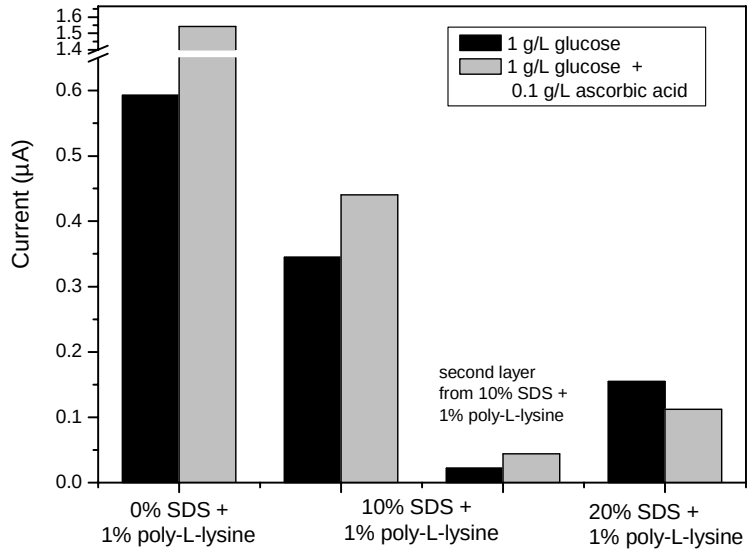


Fig. 5.22 The effect of SDS addition to the enzyme electrode response.

5.1.5.5 Polyethylene glycol (PEG)

Polyethylene glycol (PEG), which is a neutral polymer as shown in Fig. 5.23, is known as a modifier for the protection of enzymes from organic solvents (Joo et al. 1996). Yabouki and Mizutani (1995) modified glucose oxidase with polyethylene glycol, which proved to be an effective method for increasing the enzyme activity in carbon paste glucose electrodes.

The addition of 10% PEG to GOD increased glucose signals more than 3-fold (Fig. 5.24). This increase in the glucose signals may be due to the accelerated diffusion of glucose or the produced H_2O_2 (due to their sizes) through the PEG membrane.

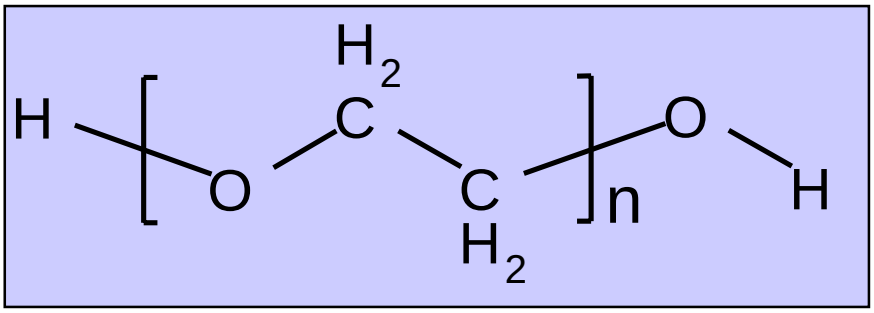


Fig. 5.23 Molecular structure for PEG.

The influence of PEG on the selectivity of GOD enzyme electrode was investigated using again ascorbic acid as a model interferant. The signals of different glucose concentrations were examined in the presence of 0.1 g/L ascorbic acid. Almost no increases of signals were observed leading to errors of 0.15, 2.4 and 3.8% as shown in Fig. 5.25. Addition of 20% PEG to GOD showed a lower response for glucose and also showed less selectivity. This is probably due to the high membrane permeability for all components present in the solution.

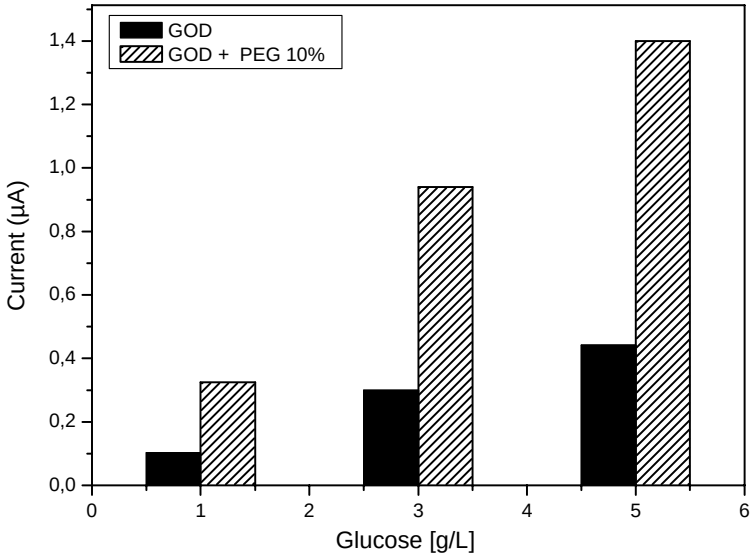


Fig. 5.24 The effect of PEG on the sensitivity of the glucose electrode.

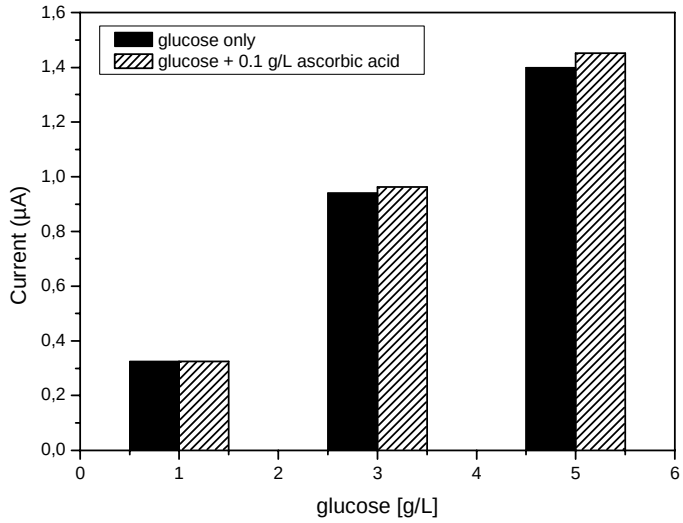


Fig. 5.25 Selectivity of the glucose electrode with 10% PEG added to GOD.

The stability of glucose electrode modified by 10% PEG was investigated after 8 and 90 days storage in Clark & Lubs buffer pH 8 at 4 ^{o}C. The remaining activity was 90% and 62% of the original activity after 8 and 90 days, respectively, the activity in the first day of preparation. The response for glucose in the presence of 0.1 g/L ascorbic acid was also investigated after 8 days. The error due to the interferences from ascorbic acid increased from 0.15 to 2.5% as shown in Fig. 5.26.

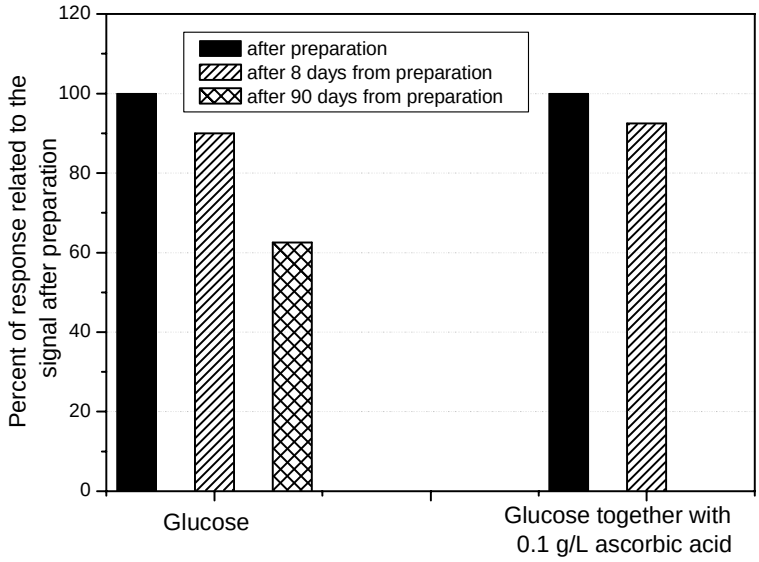


Fig. 5.26 Effect of storage on enzyme electrode with 10% PEG in the enzyme layer on the signal for 1 g/L glucose in the absence and presence of 0.1 g/L ascorbic acid.

5.1.5.6 Nafion

Nafion as a negatively charged polymer (Fig. 5.27) was used as additive to GOD to increase the negative charge in the enzyme membrane to increase the repulsion of negatively charged ions, such as ascorbic acid.

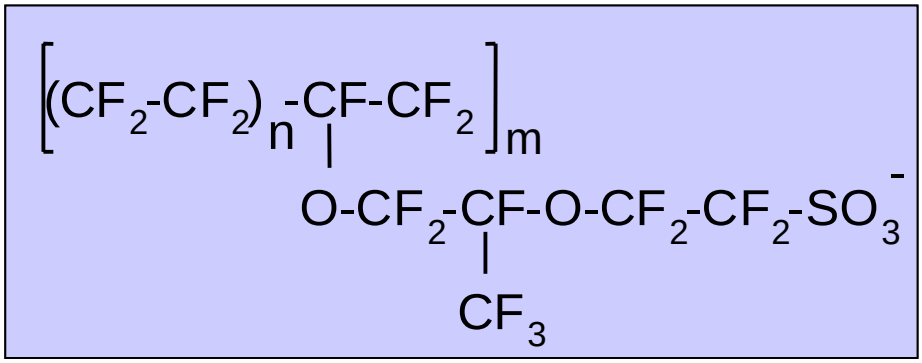


Fig. 5.27 The molecular structure for Nafion.

Two different Nafion concentrations (1 and 5%) were mixed with GOD before immobilization. Addition of 1% Nafion showed an enhancement of glucose signals, while addition of 5% Nafion increased the signal for 1 g/L glucose slightly (compared to the electrode without Nafion), whereas at higher glucose concentrations a decrease was observed as shown in Fig 5.28. This change in the sensitivity of the enzyme electrodes may be due to the presence of Nafion as enzyme additive increased the porosity of enzyme membrane and consequently the permeability of the produced hydrogen peroxide to the electrode surface increased also. While addition of 5% Nafion the enzyme layer become denser and acts as a diffusion barrier, which lowers the flux of hydrogen peroxide to the electrode surface, lower currents were obtained as mentioned before by Pan and Arnold (1996) and Gogol et al. (2000).

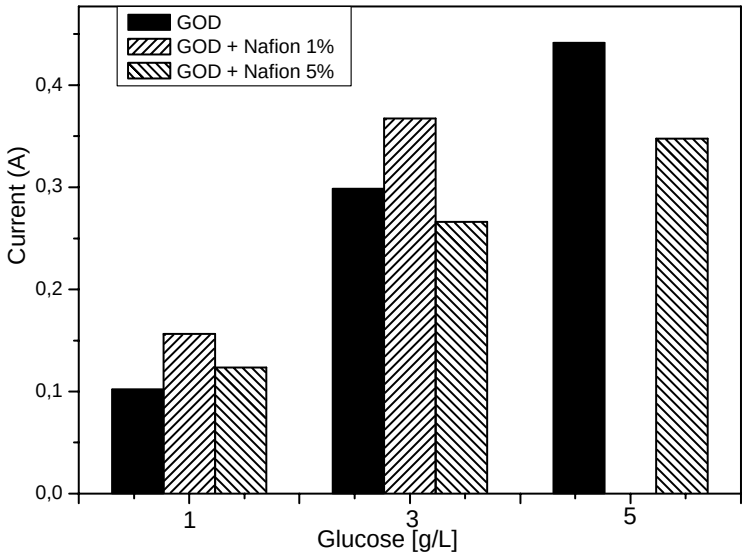


Fig. 5.28 Influence of the addition of Nafion to GOD on the signals obtained for different glucose concentrations.

Ascorbic acid was still oxidized at the electrode covered with an enzyme membrane modified by Nafion 1%. As the signals for glucose in the presence of 0.1 g/L ascorbic acid were almost doubled (Fig. 5.29). To decrease the access of ascorbic acid to the electrode, different layers made from UV paste and 1% Nafion were placed on the screen-printed electrode before or after the enzyme layer. A Nafion layer under the enzyme layer showed an enhancement of the signals for glucose in the absence and in the presence of ascorbic acid (Fig. 5.29). The addition of extra 1% Nafion layers on the top of the enzyme layer showed a marked decrease of glucose signals in the presence and absence of ascorbic acid. Addition of extra layers from Nafion increased the density of the enzyme membrane which lower the flux of hydrogen peroxide to the electrode surface (Pan and Arnold 1996; Gogol et al. 2000). Addition of 5% Nafion to the enzyme electrode increased glucose signals from 3 to 7% due to the presence of 0.1 g/L ascorbic acid (Fig. 5.30). The reduction of the influence of interferences from ascorbic acid by the addition of 5% Nafion may be due to the electrostatic repulsion between the negatively charged Nafion-enzyme membrane and ascorbic acid, which prevented its diffusion to the electrode (Chen and Tan 1995; Vaidya et al. 1996; Yang et al. 1998).

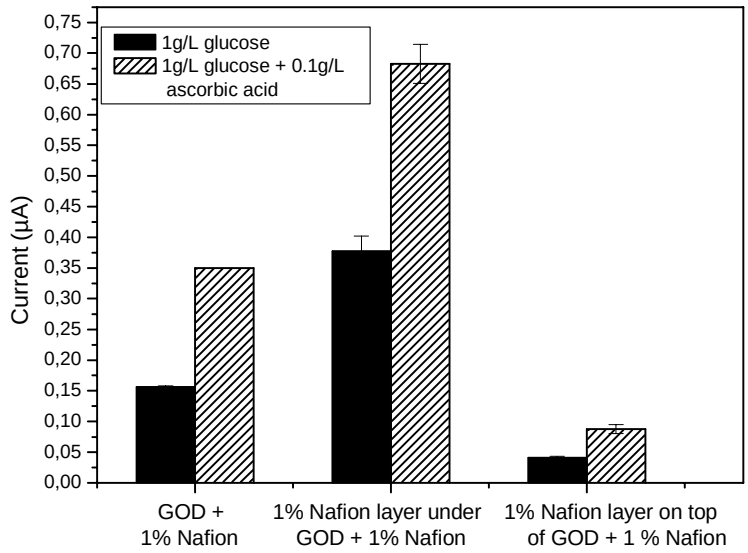


Fig. 5.29 The influence of Nafion 1% on the selectivity of GOD 60 mg/mL.

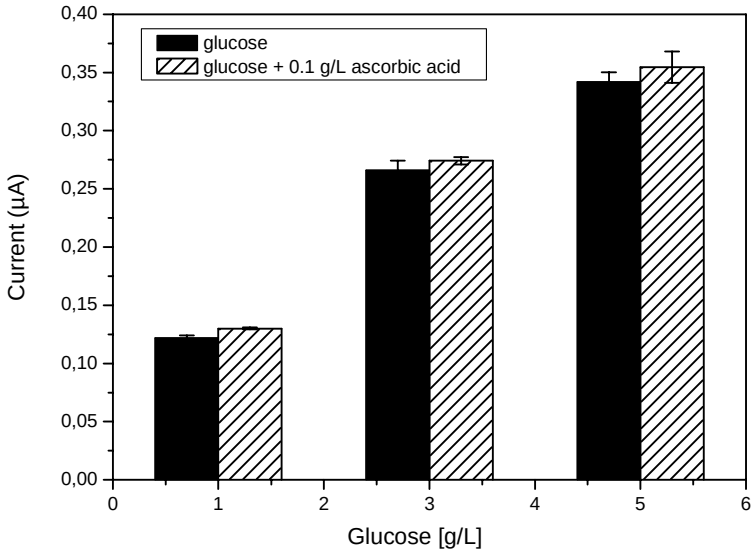


Fig. 5.30 The influence of Nafion 5% on the selectivity of a glucose electrode.

The stability and selectivity of glucose electrodes with 5% Nafion were tested after 8 and 90 days storage and it was found that the current was reduced by 9-11% (Fig. 5.31). Also the selectivity decreased, as the permeability of ascorbic acid through the membrane increased with storage time. Thus, the difference between glucose signals in the absence and presence of 0.1 g/L ascorbic acid was again increased to 16% after 8 days storage.

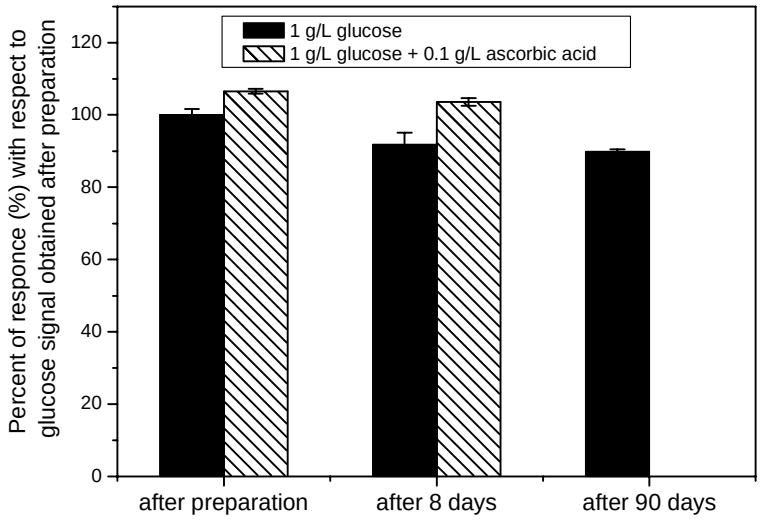


Fig. 5.31 Effect of storage time on the signals obtained with an enzyme layer comprising 5% Nafion.

5.1.5.7 Graphite

5.1.5.7.1 Electrode potential

Graphite electrodes were used for the determination of glucose using the GOD reaction together with different mediators according to equations 2.14 - 2.16 in chapter 2.2.1.4.4 (Kulys et al. 2001; Avramescu et al. 2002a; Pandey et al. 2003; Avramescu et al. 2002b). Screen-printed carbonaceous electrodes can be obtained by basically 2 different procedures: either the working electrode is printed directly from a carbon-paste (Wang 2001; Avramescu et al. 2002a; Avramescu et al. 2002b) or a screen-printed noble metal electrode, such as screen-printed Pt- or Au-electrode, is covered by a layer containing graphite particles (Sapelnikova et al. 2003). The electrochemical behaviour of a screen-printed carbon electrode for oxidation of ascorbic acid and H_2O_2 is shown in Fig. 5.32. Signals increased with increasing potential, and the electrode showed a higher sensitivity to ascorbic acid oxidation than for H_2O_2 oxidation. This behaviour was significantly different from the influence of the electrode potential in particular on glucose detection using screen-printed Pt-electrode covered by a membrane containing graphite particles and glucose oxidase (Fig. 5.33). Glucose or H_2O_2 showed higher sensitivity in the screen-printed enzyme electrode containing graphite powder than in graphite screen-printed electrode. Also glucose signals increased with increasing the applied electrode potential. Ascorbic acid showed comparable oxidation rates at both electrodes, with current increasing with increasing potentials to approximately the same maximal current. However currents at low potentials were slightly lower at the screen-

printed graphite electrode. Thus, a screen-printed Pt-electrode covered with a graphite containing membrane was to be preferred, as choice of an electrode potential above 200 mV shifts selectivity already in favour of glucose signals.

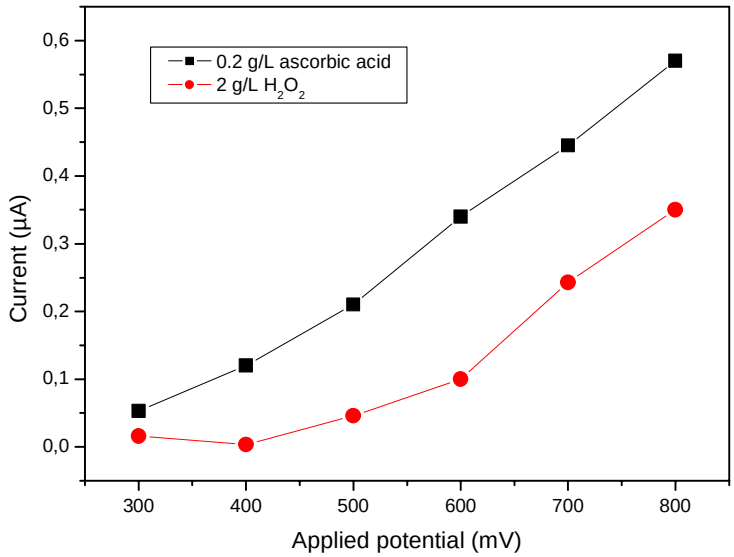


Fig. 5.32 The effect of the electrode potential on the response for 0.2 g/L ascorbic acid and 2 g/L H_2O_2 at screen-printed graphite electrode.

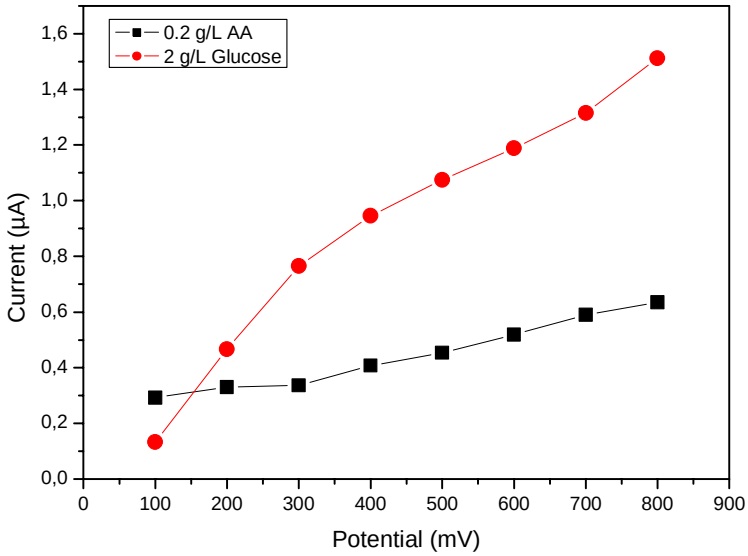


Fig. 5.33 The effect of the electrode potential on the response for ascorbic acid and glucose using platinum electrode covered by a layer containing GOD and 0.5% graphite.

5.1.5.7.2 Sensitivity and selectivity

Addition of graphite powder to the enzyme solution before immobilization led to an approximately 4 to 7-fold increase in glucose signals as shown in Fig. 5.34. This may be due

to an increased porosity of the membrane by the presence of graphite particles and an increased permeability of the enzyme membrane for H_2O_2.

The effect of graphite powder on the selectivity of the electrode was investigated by mixtures of glucose and ascorbic acid. As shown in Fig. 5.34 the presence of graphite reduced the interferences from ascorbic acid. Whereas a 4 to 7-fold increase of glucose signals was observed, no increase in signals due to the presence of ascorbic acid was found. This is compatible with the small signal for ascorbic acid oxidation at these potentials (Fig. 5.34).

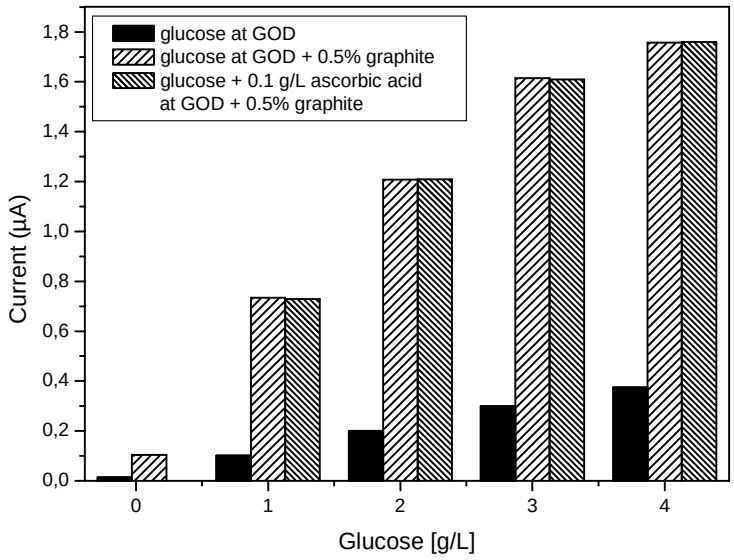


Fig. 5.34 Influence of graphite 0.5% on the sensitivity and selectivity for GOD 60 mg/mL.

5.1.5.7.3 Stability of enzyme electrode

The influence of storage in Clark & Lubs buffer at 4 ^{o}C on the activity of the enzyme electrode was studied after 8 and 90 days. The enzyme electrode delivered ~ 90% of their original signals after the studied period. The sensitivity of the electrodes towards ascorbic acid remained also unchanged, i.e. no signals were obtained for ascorbic acid (Fig. 5.35).

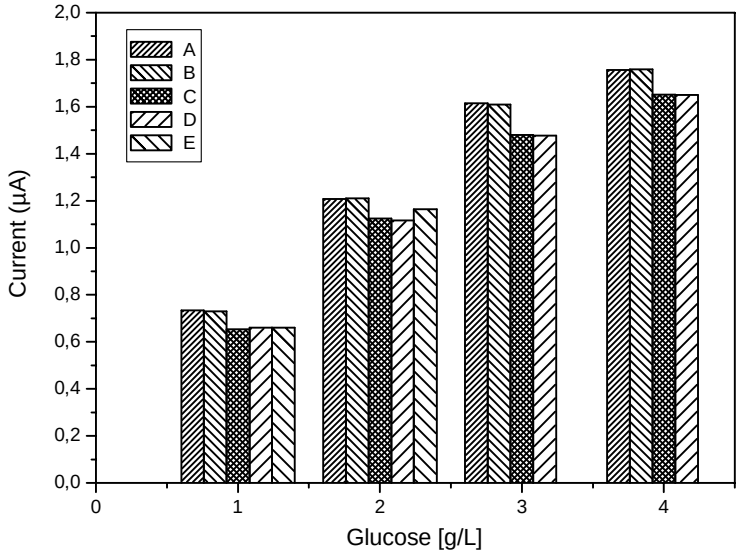


Fig. 5.35 Influence of storage for 8 days on the activity and selectivity of a glucose electrode with 0.5% graphite in the enzyme layer. (A) glucose after preparation; (B) glucose + 0.1 g/L ascorbic acid after preparation; (C) glucose after 8 days; (D) glucose + 0.1 g/L ascorbic acid after 8 days; (E) glucose after 90 days.

5.1.6 Applications

The above mentioned results led to the conclusion that glucose could be detected in the presence of ascorbic acid, if Nafion, PEG or graphite was added to the GOD membrane. With these electrodes glucose was detected in both fruit juice samples and in samples obtained from an *E. coli* fermentation. For comparison also electrodes were used with an enzyme membrane composition developed previously for long-term cultivation monitoring (BSA + poly-L-lysine) (Schumacher et al. 1999).

5.1.6.1 Determination of glucose in fruit juice samples

The contents of glucose were determined in 7 different fruit juice samples, named with their carbohydrate content in Table 5.1. As the sugar content in fruit juices varies from 2 g/L to more than 100 g/L (Belitz and Grosch 1987) and the linear ranges of the enzyme electrodes were limited from 0.1 to 2.5 g/L (Fig. 5.36), the juice samples were diluted before use. Three different dilution factors were tested (1:10, 1:50 and 1:100; except sample 1 which was diluted to 1:200, 1:300 and 1:400 due to its high carbohydrate content). The data obtained with the glucose electrodes in a FIA-system were compared to the result obtained from a reference method (YSI).

Sample	Carbohydrate content [g/L] from the manufacturer
1- Strawberry syrup (Franz ZENTIS)	Not given
2- Fruit juice with mineral water (Granini)	57.0
3- Energy drink	113.0
4- Fruit with red wine (J. Garcia Carrion, S. A)	Not given
5- Yoghurt fruit drink (Regain)	58.0
6- Carrot with honey (Weser Gold)	89.0
7- Orange juice (Weser Gold)	87.0

Table 5.1 Carbohydrate content in food samples as mentioned by the manufacturer.

5.1.6.1.1 Using GOD + poly-L-lysine + BSA electrode

The enzyme electrode containing poly-L-lysine 20% and BSA 20% in the GOD layer was used after ~ 6 months after preparation to test the influence of the dilution method. First, all standard solutions and the juice and fermentation samples were prepared in Milli-Q water. The calibration curve showed a linear range from 0.1 to 2.5 g/L (Fig. 5.36).

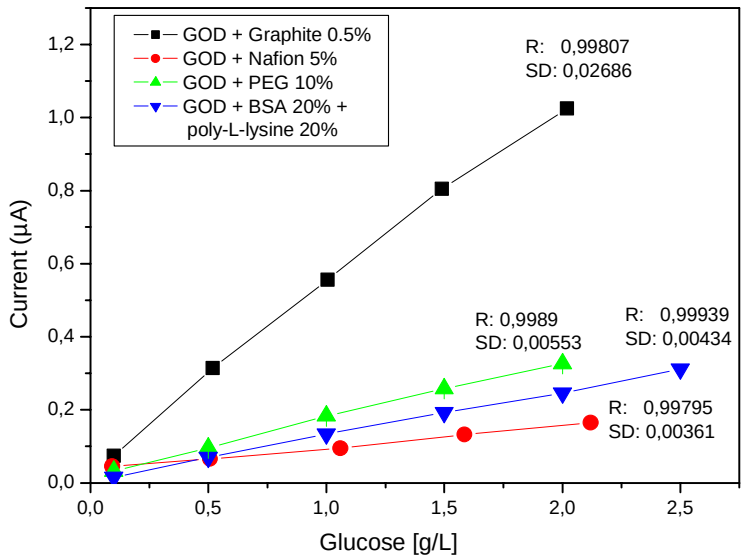


Fig. 5.36 Calibration curves for the 4 types of enzyme electrodes with different additives in the enzyme layer.

4 fruit juice samples were tested after their dilution in Milli-Q water by three dilution ratios (chapter 4.2.2) and data are shown in Fig. 5.37. A significant difference between the two methods was observed, ranging from FIA-signals being 15% lower than YSI-signals in case of sample 3 to FIA-signal being ~ 124% higher than YSI-signals in case of sample 4 (Fig. 5.38). The glucose concentrations obtained by the two systems were summarized in Table 5.2.

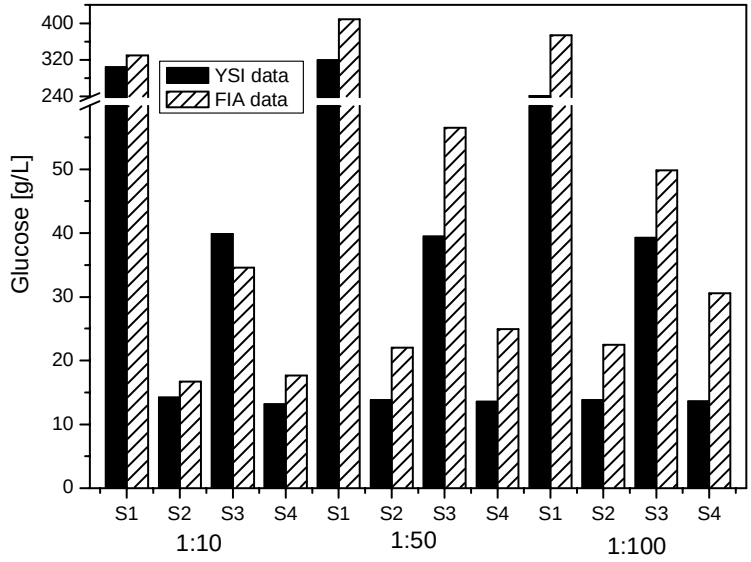


Fig. 5.37 Data obtained for glucose concentrations in 4 fruit juices using the enzyme electrode with poly-L-lysine + BSA in the enzyme layer and YSI reference method. All solutions were prepared in MilliQ-water.

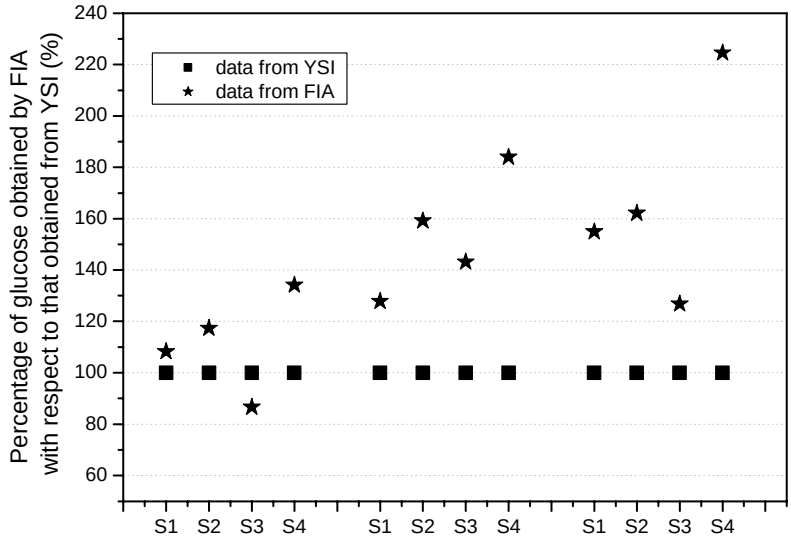


Fig 5.38 The percentage of glucose concentrations obtained with the enzyme electrode in the FIA with respect to data obtained from YSI for fruit juices. All solutions were prepared in MilliQ-water.

Alternatively, the same samples were diluted in Clark & Lubs buffer pH 8. Again, large deviations between the two systems were observed, where FIA signals were 75% to 255% of YSI-data (Fig. 5.39/5.40).

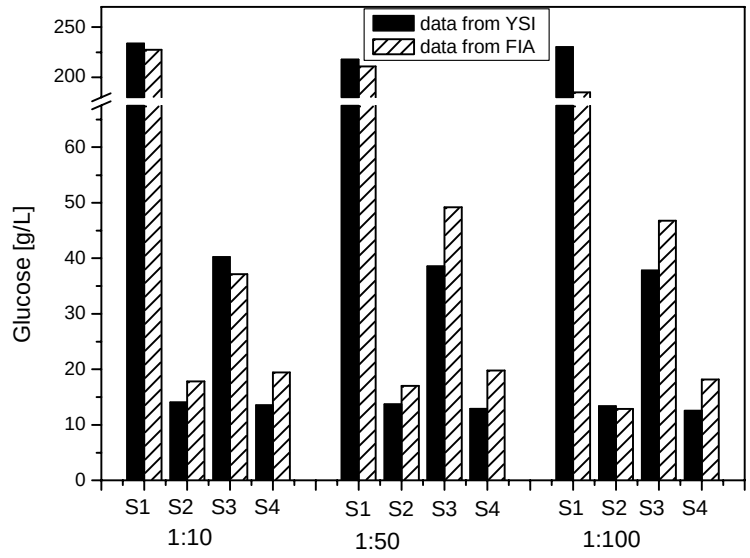


Fig. 5.39 Glucose concentrations determined in fruit juices with the enzyme electrode with poly-L-lysine and BSA in the enzyme membrane and with the reference method. All solutions were prepared in C&L buffer.

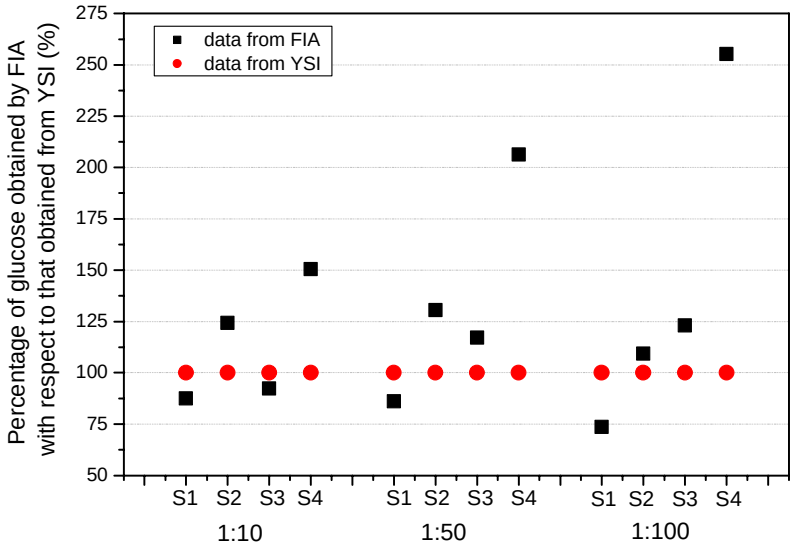


Fig 5.40 The percentage of glucose concentrations obtained with the enzyme electrode in FIA with respect to data obtained from YSI (100%) for fruit juice and fermentation samples. All solutions were prepared in C&L buffer.

	Enzyme electrode			YSI		
Sample No.	1:10	1:50	1:100	1:10	1:50	1:100
S1*	329.21	408.35	374.10	304.00	319.50	241.40
S2	16.73	22.05	22.47	14.25	13.85	13.85
S3	34.60	56.56	49.85	39.90	39.50	39.30
S4	17.64	24.93	30.55	13.15	13.55	13.60

* Sample No.1 was diluted 1:200, 1:300 and 1:400

Table 5.2 Glucose concentrations obtained from FIA and YSI. Samples were diluted with Milli-Q water.

This behaviour can be explained by considering the pH values for all diluted solutions in C&L buffer pH 8 or in Milli-Q water. As shown in Table 5.3 dilution of juices in C&L buffer showed a difference in the pH values between the standard solutions and the diluted samples. Also differences between the diluted solutions from one dilution factor to the other were obtained. Dilution of juices in Milli-Q water showed a reduced deviation in the pH values between the different dilution factors and also between the standard solutions.

Sample	Sample without dilution	Sample (1:10)	Sample (1:50)	Sample (1:100)	Standard solutions	pH
1*	3.21	6.88	7.29	7.45	0.1 g/L	8.0
2	3.55	4.7	7.19	7.62	0.5 g/L	7.94
3	3.51	4.64	6.97	7.57	1.0 g/L	7.9
4	3.37	4.54	7.56	7.84	1.5 g/L	7.87
5	3.21	5.25	7.42	7.73	2.0 g/L	7.85
6	5.31	6.65	6.96	7.9		
7	3.58	5.5	7. 66	7.878		

*Sample No. 1 was diluted by 1:100, 1:200 and 1:400.

Table 5.3 pH values for the standard and fruit juice samples diluted in C&L buffer pH 8.

Sample	Sample without dilution	Sample (1:10)	Sample (1:50)	Sample (1:100)	Standard solutions	pH
1*	3.21	4.78	4.7	4.60	0.1 g/L	5.45
2	3.55	3.66	4.77	4.78	0.5 g/L	4.94
3	3.51	3.55	3.83	4.06	1.0 g/L	4.91
4	3.37	3.79	3.68	3.84	1.5 g/L	5.02
5	3.21	3.46	3.82	4.87	2.0 g/L	4.82
6	5.31	5.60	5.65	5.37		
7	3.58	3.58	3.90	4.28		

*Sample No. 1 was diluted by 1:100, 1:200 and 1:400.

Table 5.4 pH values for the standard and fruit juice samples diluted Milli-Q water.

From the above results and from the preliminary tests in the detection of glucose in food and cultivation samples using GOD electrode modified by different additives we found that the results of samples diluted with water showed less deviations between FIA and YSI, in particular on overestimation.

5.1.6.1.2 Nafion electrode

Figs. 5.41 - 5.43 show the data obtained for the fruit juice samples (Table 5.1) diluted to various degrees by Milli-Q water using the enzyme electrode with Nafion (5%) in the enzyme membrane and for comparison the data from YSI.

Using 1:10 dilution samples 2, 4, 5, and 7 showed the best correlation between the two detection methods, while sample 6 showed the highest deviation (Figs. 5.41, 5.42). Fig. 5.43 represents the relation between the data obtained using the screen-printed enzyme electrode and the standard YSI. The statistical tests showed no significant difference between the two systems with t-value of 0.139 with 0.05 degree of freedom (Fig. 5.43).

For samples diluted 1:50 sample 6 showed again the highest deviation (50%). Samples 1 and 4 showed deviation by ~ 22 and 13% respectively, while the other samples showed less than $\pm$5%. Also the statistical tests for the obtained results by the two measurement systems showed no significant difference with 0.223 t-value using 0.05 degree of freedom (Fig. 5.43).

1:100 dilution factor showed the highest correlation between the obtained results from the two detection systems (Fig. 5.41) where less than 6% deviations were obtained (Fig 5.42).

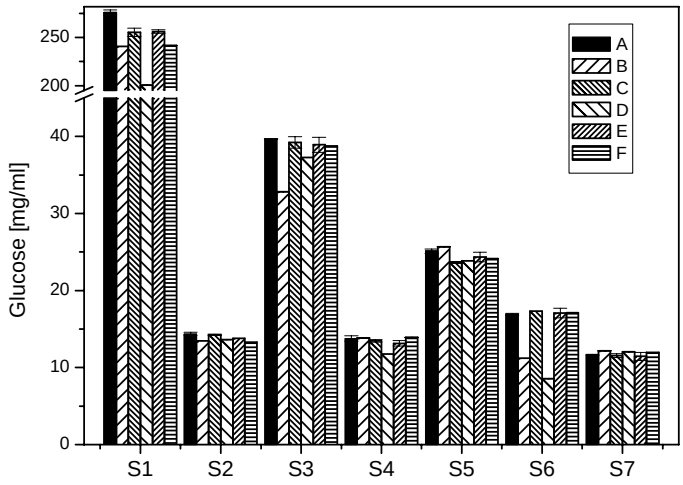


Fig. 5.41 Glucose concentrations in fruit juices determined with the enzyme electrode with Nafion in the enzyme membrane and with the YSI. (A) YSI (1:10); (B) FIA (1:10); (C) YSI (1:50); (D) FIA (1:50); (E) YSI (1:100); (F) FIA (1:100).

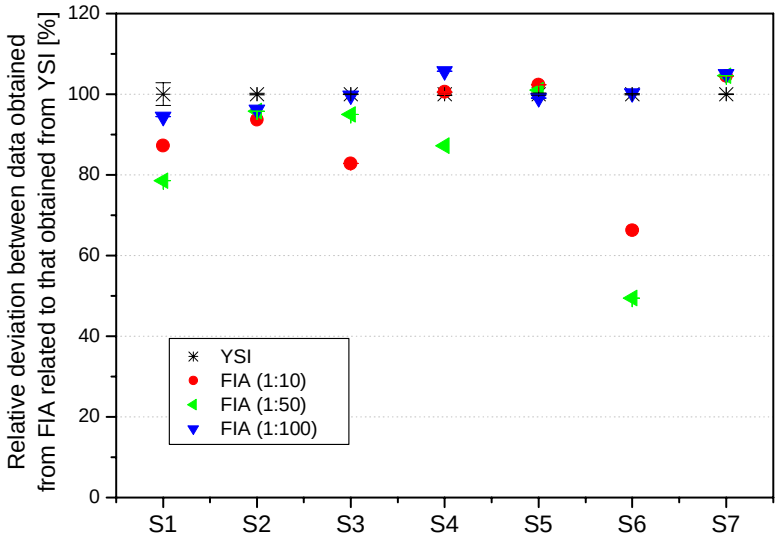


Fig. 5.42 Relative deviation between data obtained from FIA with to YSI (100%) using different degrees of dilution.

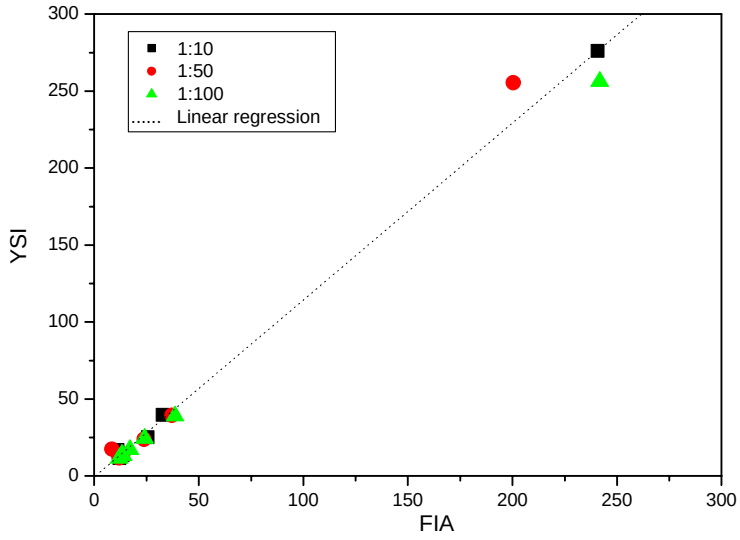


Fig. 5.43 Correlation between the data obtained from the enzyme electrode modified with Nafion 5% in FIA and those obtained by YSI.

By application of t-test to the obtained results the t-value showed that there is no significant difference between the two used systems (t-value 0.908, using 0.05 degree of freedom). The actual data obtained from the two detection systems are summarized in Table 5.5.

	Enzyme electrode			YSI		
Sample (No.)	1:10	1:50	1:100	1:10	1:50	1:100
S1*	240.82	200.50	241.84	276	25.30	256.20
S2	13.44	13.65	13.27	14.35	14.25	13.80
S3	32.83	37.24	38.76	39.65	39.22	38.90
S4	13.83	11.73	13.90	13.75	13.45	13.15
S5	25.69	23.85	24.11	25.10	23.62	24.35
S6	11.20	8.55	17.09	16.90	17.30	17.05
S7	12.17	12.05	11.99	11.65	11.525	11.42

* Sample No.1 was diluted 1:200, 1:300 and 1:400

Table 5.5 The actual data obtained for glucose in different food samples using FIA (with enzyme electrode in the Nafion in the enzyme membrane) and YSI.

5.1.6.1.3 PEG electrode

Glucose was determined in the fruit juice samples using the enzyme electrode with 10% PEG in the enzyme membrane. The resultant data and the data obtained from FIA and YSI are shown in Figs. 5.44 - 5.46.

For juices diluted by 1:10, data obtained from the PEG enzyme electrode and YSI high correlation with deviations less than 6%, except sample 3 which showed a higher deviation (~17%) (Fig. 5.45). The t-test was applied to the obtained results, where it showed that there was no essential difference between the two systems (t-value = 0.0685) using 0.05 degree of freedom.

The data obtained for the 1:50 dilution showed a higher deviation than those obtained from 1:10 dilution (Fig. 5.45). Again sample 3 showed a deviation by ~ 16%, and sample 6 gave glucose content 22.3% lower than that obtained from YSI, while the other samples showed deviations of less than 11%. The obtained t-value is 0.00825 using 0.05 degree of freedom.

With 1:100 dilution for the juices, the obtained data from FIA were highly correlated to YSI. Samples 1, 4, 5 and 6 were showed less than 5 % deviations, while sample 2 showed a ~ 24% deviation (Fig. 5.45). The statistical t-test was applied to the obtained results and showed t-value of - 0.01915 (with 0.05 degree of freedom), where is no essential difference between the data from the two systems (Fig. 5.46). The actual data obtained from the two detection systems are summarized in Table 5.6.

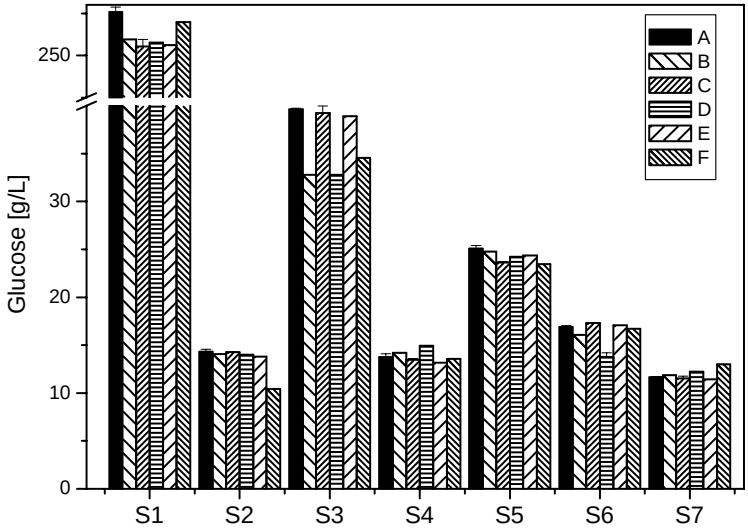


Fig. 5.44 Glucose concentrations in fruit juices determined with an enzyme electrode with 10% PEG in the enzyme membrane. (A) YSI (1:10); (B) FIA (1:10); (C) YSI (1:50); (D) FIA (1:50); (E) YSI (1:100); (F) FIA (1:100).

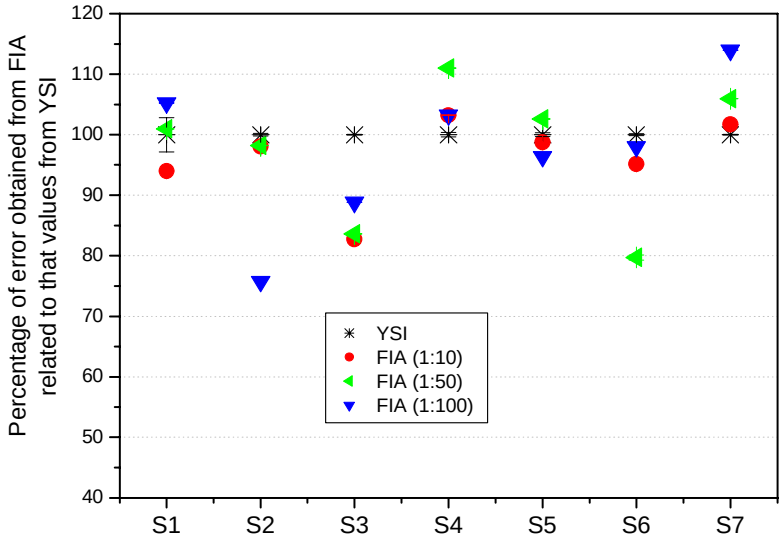


Fig. 5.45 The percentage of errors between the obtained data from FIA with PEG electrode and YSI (100%).

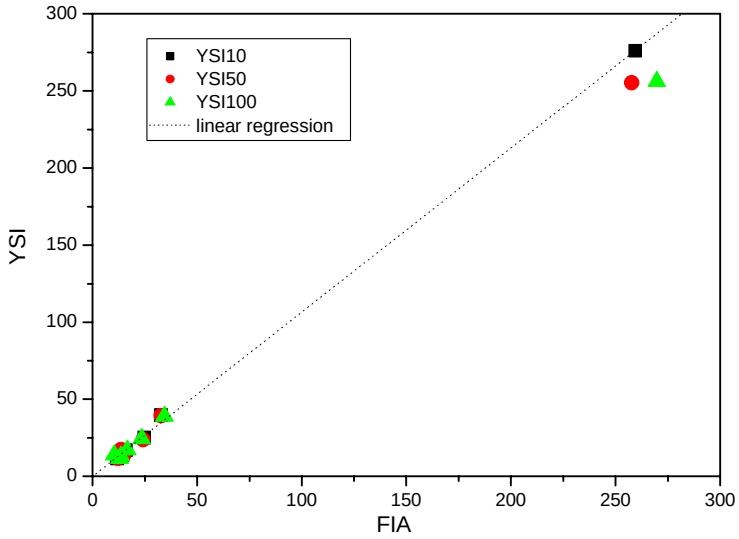


Fig. 5.46 Correlation between the data obtained from the enzyme electrode modified with 10% PEG in FIA and those obtained from YSI.

	Enzyme electrode			YSI		
Sample (No.)	1:10	1:50	1:100	1:10	1:50	1:100
S1*	259.47	257.71	269.73	276.00	255.30	256.20
S2	14.07	13.99	10.45	14.35	14.25	13.80
S3	32.80	32.79	34.55	39.65	39.22	38.90
S4	14.19	14.93	13.58	13.75	13.45	13.15
S5	24.78	24.24	23.47	25.10	23.62	24.35
S6	16.08	13.79	16.71	16.90	17.30	17.05
S7	11.85	12.21	13.02	11.65	11.52	11.42

* Sample No.1 was diluted 1:200, 1:300 and 1:400

Table 5.6 The actual data obtained for glucose in different food samples using FIA (with enzyme electrode in the PEG in the enzyme membrane) and YSI.

5.1.6.1.4 Graphite electrode

Glucose concentrations in juice samples diluted 1:10 determined with the enzyme electrode with 0.5% graphite in the enzyme membrane and the data obtained from YSI are shown in Figs. 5.47 – 5.49. All samples showed good correlations between the two detection systems with deviation less than 10% except sample 3 which showed the higher deviation (44%). The statistical t-test showed that no essential difference between the two method of measurements with t-value of 1.0069 with 0.05 degree of freedom.

Using 1:50 dilution, the obtained data showed a good correlation with a less than 10% deviation. The t-test was applied to the obtained data, using 0.05 degree of freedom a t-value of - 0.0282 was obtained, which indicates that there is no essential difference between the two systems of detection.

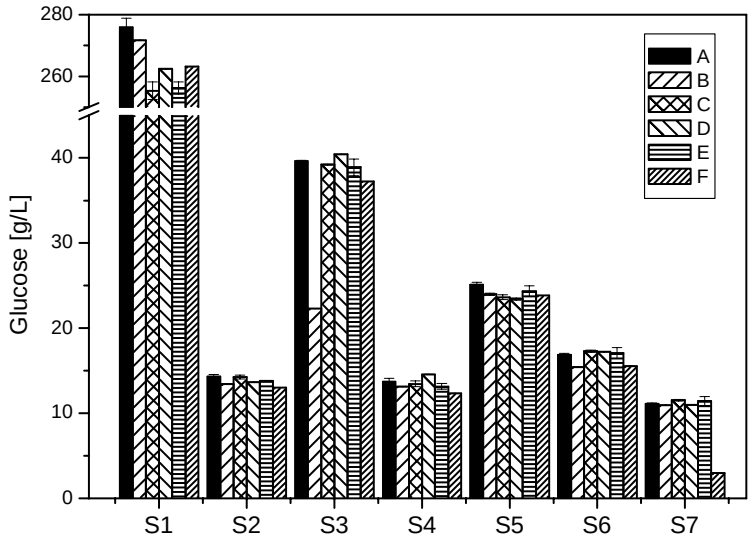


Fig. 5.47 Glucose concentrations in fruit juices determined with an enzyme electrode with 0.5% graphite in the enzyme membrane: A) YSI (1:10); B) FIA (1:10); C) YSI (1:50); D) FIA (1:50); E) YSI (1:100); F) FIA (1:100).

Sample 7 showed the highest deviation between the graphite-enzyme electrode and YSI data when the samples were diluted 1:100. Other samples showed a good correlation with a deviation lower than 10% (Figs. 5.47 - 5.49). The relation between the glucose concentrations obtained with the two systems is shown in Fig. 5.49. A linear relation was obtained with a t-value of 0.01199 with 0.05 degree of freedom, thus no essential difference was observed. Table 5.7 collect the actual data obtained for glucose in the tested food samples from FIA and YSI.

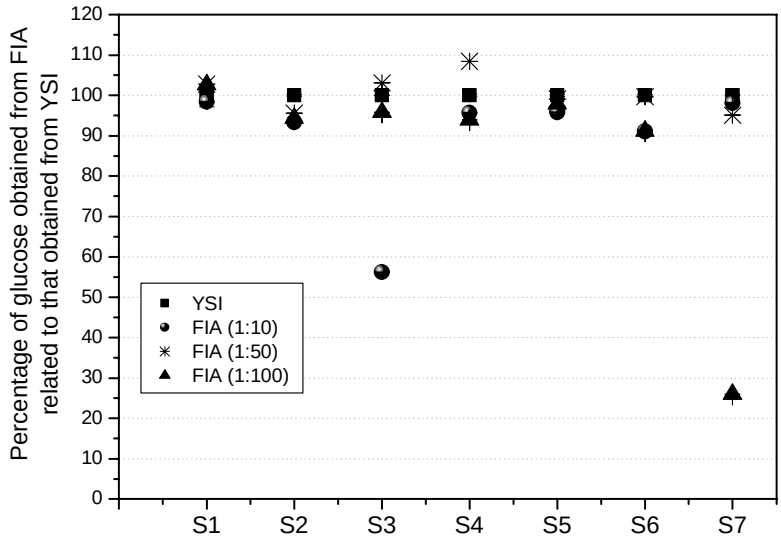


Fig. 5.48 The percentage of errors between the obtained data from FIA with graphite electrode and YSI (100%).

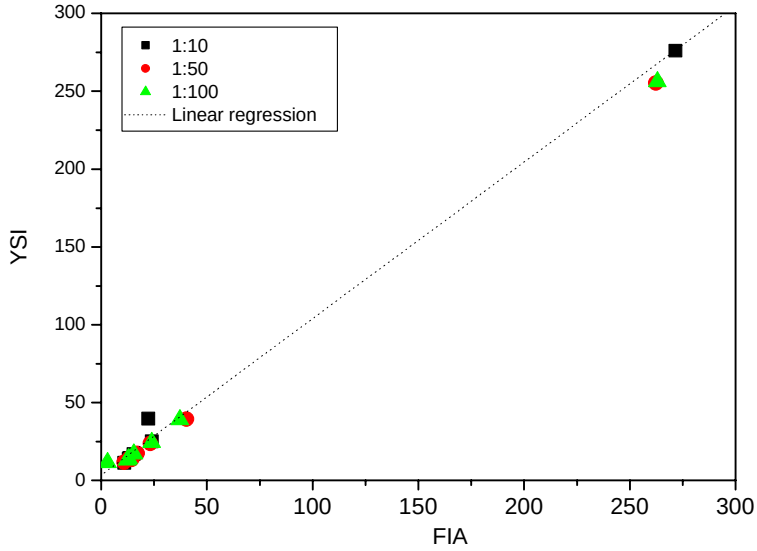


Fig. 5.49 Correlation between the data obtained from FIA using enzyme electrode modified with graphite 0.5% and those obtained by YSI.

	Enzyme electrode			YSI		
Sample No.	1:10	1:50	1:100	1:10	1:50	1:100
S1*	271.61	262.45	263.09	276.00	255.30	256.20
S2	13.39	13.62	13.02	14.35	14.25	13.80
S3	22.29	40.44	37.25	37.65	39.22	38.90
S4	13.15	14.58	12.35	13.75	13.45	13.15
S5	23.99	23.41	23.84	25.10	23.62	24.35
S6	15.39	17.24	15.54	16.90	17.30	17.05
S7	10.94	10.96	2.97	11.15	11.25	11.42

* Sample No.1 was diluted 1:200, 1:300 and 1:400

Table 5.7 The actual data obtained for glucose in different food samples using FIA (with enzyme electrode in the graphite in the enzyme membrane) and YSI.

In Table 5.8 the statistical data are collected obtained for the linear relations between the results obtained from the different enzyme electrodes and YSI and also the obtained t-values.

		R	t-value	SD
* GOD + Nafion 5%	1:10	0.999	0.139	3.098
	1:50	0.999	0.223	5.243
	1:100	0.999	0.043	0.665
* GOD 60 + PEG 10%	1:10	0.999	0.068	2.336
	1:50	0.999	- 0.008	0.335
	1:100	0.999	- 0.019	2.596
*GOD + Graphite 0.5%	1:10	0.846	1.007	6.271
	1:50	0.999	0.028	0.679
	1:100	0.999	0.0198.	2.838
* GOD+ BSA + poly- L-lysine	1:10	0.999	- 0.951	5.951
	1:50	0.999	0.262	1.325
	1:100	0.999	0.416	6.651

* Sample No.1 was diluted 1:200, 1:300 and 1:400.

Table 5.8 Statistical data resulted from plotting the obtained data from FIA against the data from YSI.

5.1.6.2 Determination of glucose in cultivation samples from an E. coli cultivation

Glucose was used as carbon source in *E. coli* cultivations. The amounts of remaining glucose during the cultivation process were determined using enzyme electrodes with different additives in the enzyme membrane. The initial glucose concentration was 20 g/L. The samples were taken after different cultivation times from 2 to 11 h and were diluted 1:10 by Milli-Q water. For comparison the data from a reference method (YSI) were used.

The obtained data are shown in Figs. 5.50 - 5.52. The obtained data using BSA and poly-L-lysine in the enzyme membrane were higher than the data from YSI with a deviation higher than 10%.

Using enzyme electrode with Nafion, the obtained data showed a good correlation in the first 7 h of cultivation with a deviation of less than 10%, while for higher cultivation times the deviation increased 18 and 86% after 10 and 11 h, respectively.

Data obtained with the enzyme electrode with PEG were highly correlated to those obtained from YSI in all cultivation times with small deviation lower than 2.5% (Fig. 5.51).

The remaining amounts of glucose were also determined in the cultivation samples using the enzyme electrode with graphite in the enzyme membrane. The obtained data are shown in Fig. 5.50, showing a deviations between both methods lower than 10%. The data obtained from FIA and YSI are collected in Table 5.9.

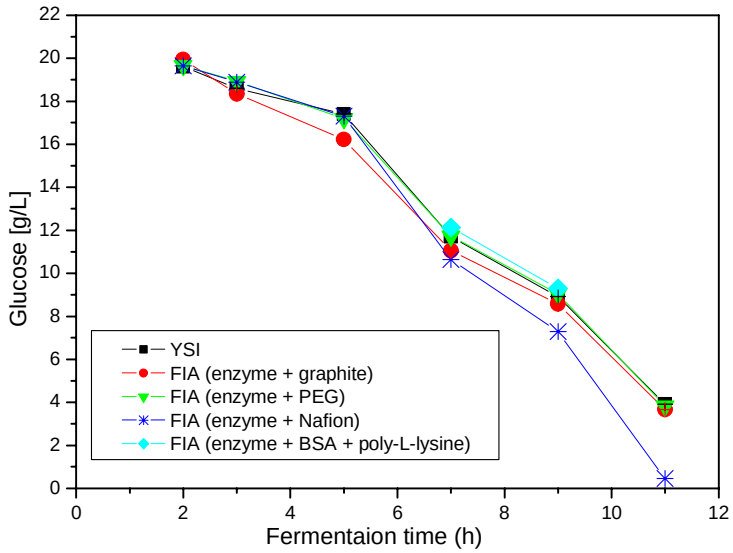


Fig. 5.50 Glucose concentrations obtained from the cultivation samples using enzyme electrode with different additives in the enzyme membrane and from YSI.

A linear relation was obtained between the data resulting from enzyme electrodes with different additives in the enzyme layer and YSI (Fig. 5.52). All statistical data are collected in Table 5.10. Linear relations were obtained and the t-test showed no significant difference between the two systems of detection.

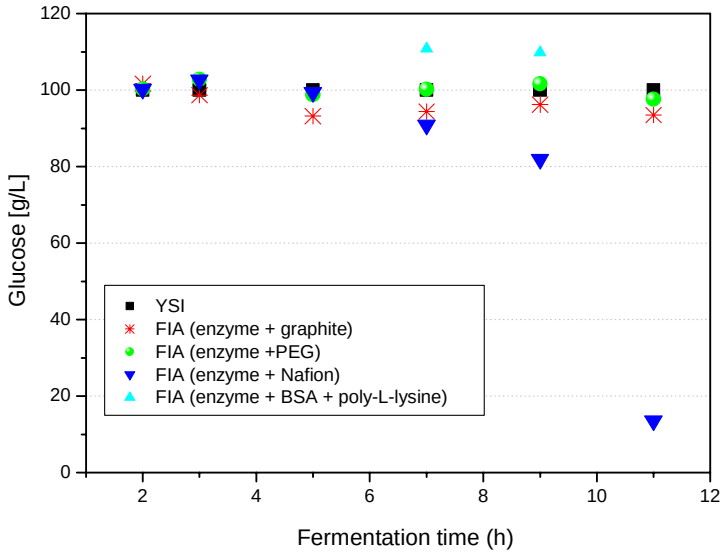


Fig. 5.51 The percentage of errors between the obtained data from FIA (using enzyme electrode with different additives) and the data from YSI.

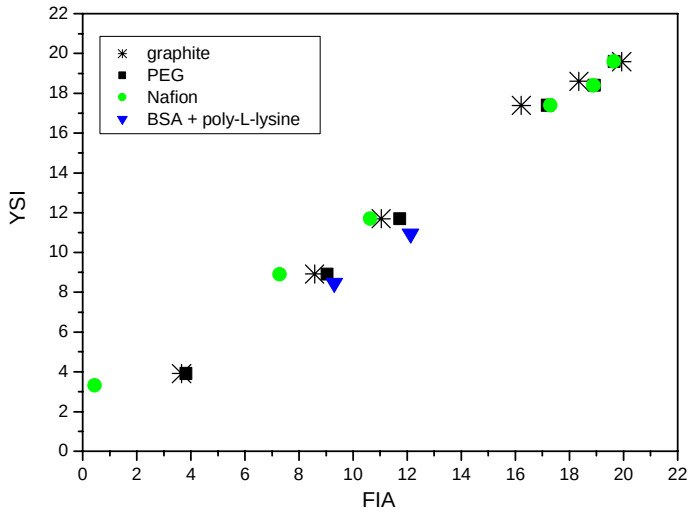


Fig. 5.52 Correlation between the obtained data from FIA (using enzyme electrode with different additives) and the data obtained from YSI.

Time (h)	YSI [g/L]	Enzyme electrode [g/L]			
		Graphite	PEG	Nafion	Poly-L-lysine + BSA
2	19.60	19.93	19.66	19.64	
3	18.60	18.35	18.92	18.89	
5	17.40	16.22	17.73	17.30	
7	11.70	11.04	11.73	10.63	12.13
9	8.91	8.573	9.04	7.29	9.30
11	3.92	3.66	3.82	0.45	

Table 5.9 The actual data obtained for glucose in cultivation samples (all samples were diluted 1:10) using FIA and YSI.

	GOD 60 mg/mL + Nafion 5%	GOD 60 mg/mL + PEG 10%	GOD 60 mg/mL + Graphite 0.5%
R	0.999	0.999	0.999
t- value	0.246	0.020	0.098
SD	0.258	0.205	0.430

Table 5.10 Statistical data resulted from the relation between FIA and YSI in the determination of glucose in cultivation samples (all samples were diluted 1:10).

5.1.7 Conclusion

In the determination of glucose using amperometric enzyme electrodes, the addition of BSA, Gafquat 755N and poly-L-lysine to GOD enhanced the sensitivity of the enzyme electrodes but showed only little selectivity towards the interferant materials. This enhancement of the sensitivity and the low selectivity were due to the improved permeation of H_2O_2 produced through the GOD-catalyzed reaction as well as of interferents (Mizutani et al. 1998). Addition of the negatively charged polymer Nafion showed a reduced sensitivity but higher stability up to 90 days. Addition of the neutral polymer PEG did not eliminate completely the influence of interferences, but showed a higher sensitivity for the prepared enzyme electrode. Graphite powder was added during the immobilization and could eliminate the influences produced from the presence of ascorbic acid, together with increases of the sensitivity for glucose detection from 4 to 7-folds. The obtained stabilities, sensitivity and selectivity from the various additives in addition to the effects from other additives as reported in literature are collected in Table 5.11.

The prepared glucose oxidase electrodes modified by Nafion 5%, PEG 10% or graphite 0.5% could be used for the determination of glucose in food samples and in bioprocess samples.

Additive	Immobilization method	Stability	Selectivity	Sensitivity	Reference
Poly-L-lysine + poly-4-styrenesulfonate	Cross-linking	2 months	Ascorbic acid 7% Uric acid 4% Acetaminophen 15%	--	Mizutani et al. 1998
Protein Based Stabilizing Agent (PBSA)	Cross-linking	3-folds increase compared to soluble enzyme	--	--	Gouda et al. 2001
Nafion	Entrapment	3 months	Ascorbic acid 5.6% Uric acid 1.2% Acetaminophen 1%	--	Yang et al. 1998
Nafion	Entrapment	Constant till 15 days, 80% till 100 days	Ascorbic acid 0% Uric acid 1120-120% Acetaminophen 246-350%	Low sensitivity	Xu et al. 2002
Nafion + Tetrathiafulvalene	Cross-linking	2 months	No effect from ascorbic acid	High sensitivity	Liu and Deng 1995
DEAE-dextran	Adsorption	Over 3 months	---	High sensitivity	Gavalas et al. 1998
DEAE-dextran	Adsorption	6 months 50% after 9 months	--	4.39 ± 0.04 µA/mM	Gavalas et al. 2000
DEAE-dextran Lactitol, or Gafquat 755N	Cross-linking	Increased	--	--	Gibson et al. 1996
Gafquat 755N	Entrapment	3 months	250% ascorbic acid	4-folds	Current work
Nafion 5%	Entrapment	3 months	0.05-5% ascorbic acid	Less senstivity	Current work
PEG 10%	Entrapment	3 months	0.15-3.75% ascorbic acid	3-folds	Current work
Graphite 0.5%	Entrapment	3 months	0.05%	7-folds	Current work

Table 5.11 The effect of some additive on the stability, sensitivity and selectivity of GOD electrode.

5.2 Determination of glucose using microchip capillary electrophoresis

5.2.1 General description of microchips

Glass microchips (Fig. 4.4) were fabricated from Schott Borofloat glass, PMMA chips (Fig. 4.4) were fabricated from poly(methylmethacrylate) polymer, and ceramic microchips (Fig. 4.5) were fabricated by Low Temperature Cofired Ceramic (LTCC) technology. The company, dimensions, channel cross-section, separation and injection channel lengths for each type of microchips were collected in Table 5.12.

Chip material	Company	Dimensions wide x length x height (mm)	Channel cross section (µm)	Separation channel length (mm)	Injection channel length (mm)
Glass	Micralyne	16 x 95 x 2.2	20x50 Half-circle	85	8
PMMA	AMT Jena	16 x 95 x 2.2	20x50	85	8
Ceramic	VIA electronic	19 x 58.5 x 1.0	94x94	49	8

Table 5.12 Description of different microchips.

5.2.2 Characterization of microchips

The electric field applied to a capillary filled with a conductive liquid leads to an electric current according to the Ohm's law. Flow of electric currents is related to the production of heat, the so-called Joule's heat, and, thus, to an increase in temperature. To maintain constant and reproducible experimental conditions an uncontrolled heating of liquid is to be avoided. Moreover, increasing temperatures affect separation quality and mobilities of compounds. Thus, the current within the capillary has to be limited. It is dependent on the diameter of the capillary, ionic strength of the buffer and the electric field. Recording the current in dependence of the electric field should result in a straight line according to Ohm's law, with the slope being related to the resistance of the field capillary. Increasing temperature lead to a decreasing resistance of the buffer, and thus to a deviation from the linear relationship, if too high voltages are chosen, i.e. the current is too high. This threshold is dependent on experimental conditions, among others on the material and size of the capillary.

Mobility of compounds is affected by the electrosmotic flow (see chapter 2.2.2.2), which is dependent on the Zeta-potential, and thus, using constant buffer conditions, on the surface charge density on the inner capillary surface. As major contributions originate from the ionization of surface groups, the surface charge density is dependent on the chemical

composition of the capillary surface, i.e. on the capillary material and required processing procedures. The EOF can be determined via the migration time of an electrically neutral compound. Thus, chips were characterized by current-voltage curves and EOF measurements.

5.2.2.1 Glass microchips

5.2.2.1.1 Current-Voltage-Relationship

Different high voltages ranged from 0.1 kV to 7.5 kV were applied to double T-intersection glass microchips and the resultant currents in the separation channel were recorded. The current increased with increasing the applied high voltage with a linear range from 0.1 kV to 6 kV with a correlation coefficient of 0.999, 66.31 SD and 205 MΩ resistance (Fig. 5.53).

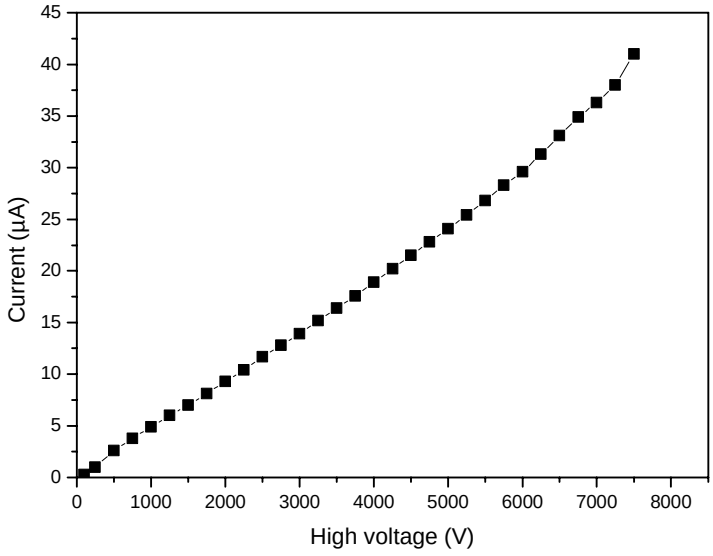


Fig. 5.53 Current-voltage relationship for double T-intersection glass microchips.

5.2.2.1.2 EOF determination

The EOF for glass microchips was measured using phosphate and Clark & Lubs buffers (10 mM pH 8). H_2O_2 was used as a neutral marker. The obtained values are collected in Table 5.13. It was observed that the EOF was higher when a phosphate buffer was used as a running buffer.

	High voltage (V)	Migration time (min)	EOF (mm/min)	Mobility (mm^2/V min)
Phosphate buffer	3000	1.17 ± 0.017	72.65	2.06
	2000	2.67 ± 0.033	31.83	1.35
	1000	3.71 ± 0.033	22.91	1.95
Clark & Lubs buffer	1000	5.4 ± 0.05	15.44	1.31

Table 5.13 The obtained EOF and mobilities for glass microchips.

5.2.2.2 Poly(methylmethacrylate) microchips (PMMA)

The organic polymer poly(methylmethacrylate) (PMMA) was used for the fabrication of capillaries and microchips (Schneider and Engelhardt 1998; Song et al. 1999; Wang et al. 2002; Lee et al. 2001). The chemical structure for PMMA is shown in Fig. 5.54.

Two different PMMA microchips were tested, according to the type of injection channel, the first with a simple T-intersection (or simple cross) channel and the second with a double T-intersection channel as illustrated in Fig. 5.55.

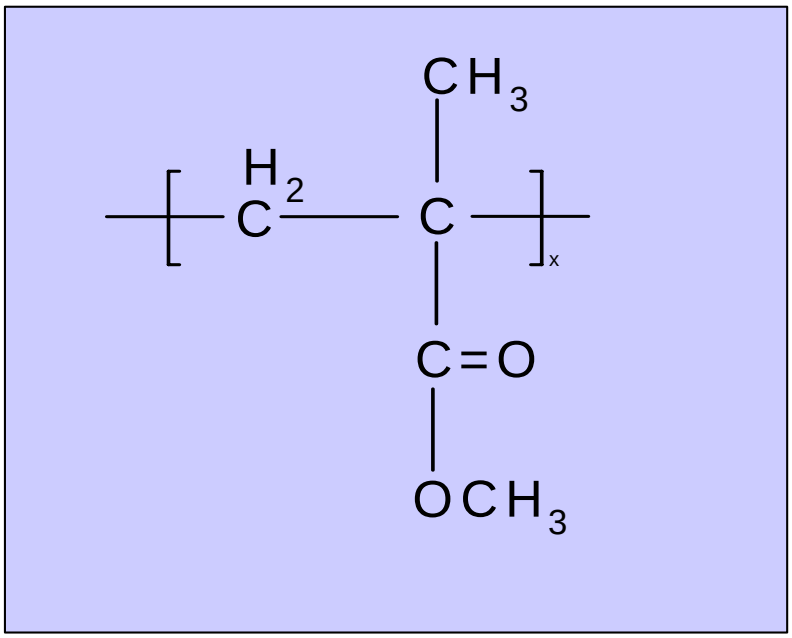


Fig. 5.54 The chemical structure for PMMA polymer.

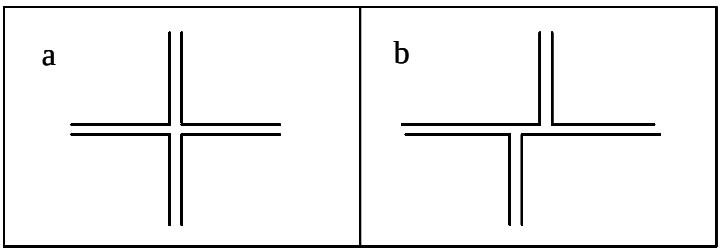


Fig. 5.55 The layout for (a) single T and (b) double T injection channels.

PMMA is the most commonly used polymer materials for the production of microanalytical systems due to the low cost and the ease of manipulation compared to silica-based substrates. Incorporation of nitrogen and oxygen during the fabrication process of polymer microfluidic devices can give rise to amino, hydroxyl, carboxylic, or phenolic functional groups at the surface. These functional groups are thought to play an important role in the EOF (Roberts et al. 1997). The EOF was reversed by the modification of PMMA surfaces by amino groups compared to unmodified substrates (Henry et al. 2000). Martynova et al. (1997) reported that no adsorption on the PMMA surface was found for analytes such as carboxyflourescein or proteins, but the EOF was found in the PMMA microchips fabricated by the imprinting method. Recently, Dang et al. (2003) reported that APTS-labeled oligosaccharides (APTS = 8-aminopyrine-1,3,6-trisulfonate) adsorbed very strongly to the surface of PMMA microchips. Amines and surfactants such as CTAB and SDS did not show observable effects on suppressing this adsorption, but neutral polymer additives with polyhydroxyl groups such as hydroxyethylcellulose, methylcellulose and hydroxypropylmethylcellulose suppressed the hydrophobic interaction between the APTS and the PMMA channel surface. Nonionic surfactants such as Brij 35, Brij 78 and Brij 76 were used for PMMA surface coating in the separation of double-stranded DNA fragments (Song et al. 1999).

5.2.2.2.1 Current-Voltage-Relationship

The relationship between current and applied high voltage was recorded according to chapter 4.3.6.1. Data are shown in Fig. 5.56 for the single T-intersection chips. By increasing the electric field strength the current increased. A linear region was observed up to a high voltage of + 6.0 kV. Higher voltages led to Joule heating and thus to a stronger increase of the current. The resulting resistance obtained for two independent experiments is given in Table 5.14.

The same behaviour was observed in PMMA microchips with a double T-injection channel. The current increased linearly by increasing the applied high voltage to 6.0 kV (Fig. 5.57). At higher voltages the current increased stronger due to Joule heating. The resulting resistance is given in Table 5.14.

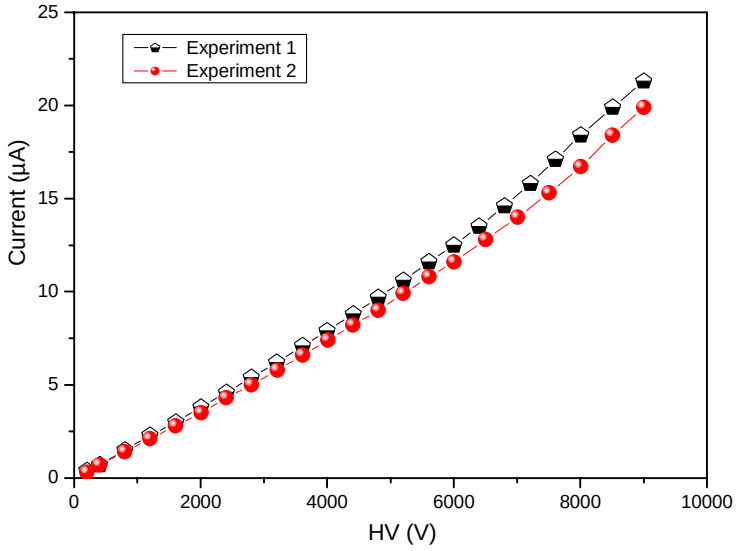


Fig. 5.56 Current-voltage relationship for single T-intersection PMMA microchips.

	Single T-intersection			Double T-intersection			
	1	2	Mean	1	2	3	Mean
Resistance (MΩ)	515.4	480.2	497.8	580.3	523.9	526.9	543.7
SD	74.220	78.750	76.485	53.850	70.810	62.760	62.473
R	0.999	0.999	0.999	0.999	0.999	0.999	0.999

Table 5.14 The resistance and statistical data the for PMMA microchip.

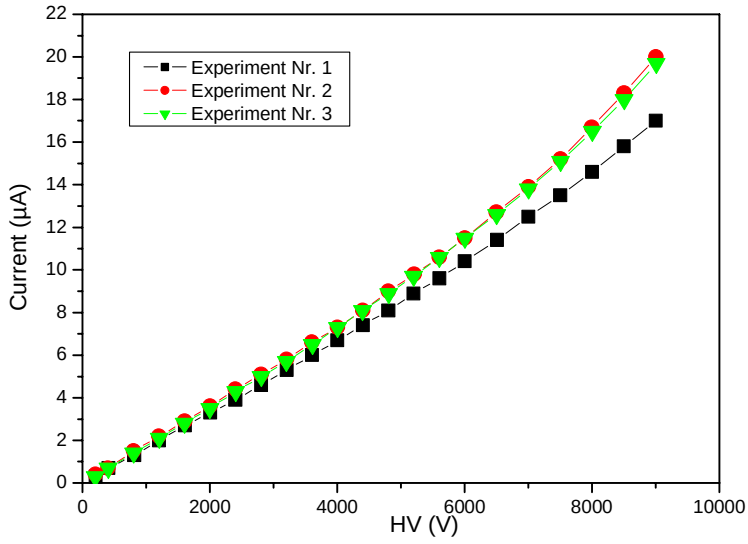


Fig. 5.57 Current-voltage relationship for double T-intersection PMMA microchips.

5.2.2.2.2 EOF determination

EOF measurements were carried out according to chapter 4.3.6.2. The influence of different high voltages on the EOF was tested, where by increasing the high voltage from + 600 V to + 2000 V the transportation time decreased from 260 s to 570s and the EOF increased (Fig. 5.58). The preliminary effect of the negative surfactant SDS was also examined using + 600 V, where the addition of 1 mM enhanced the EOF (Fig. 5.58).

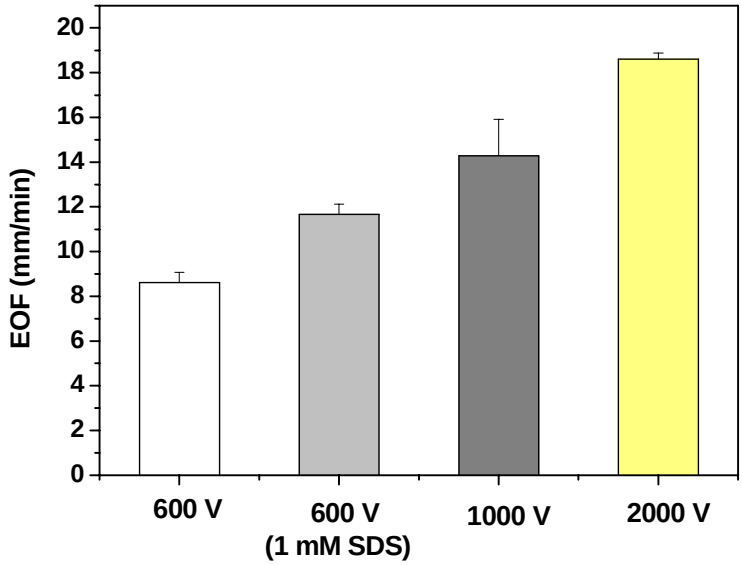


Fig. 5.58 EOF values obtained from PMMA microchips.

Thus in the following experiments 2 kV were applied as a high voltage, which avoids Joule heating by sufficiently fast transportation.

5.2.2.3 Ceramic microchips

5.2.2.3.1 Current-Voltage-Relationship

The third type of microchips were ceramic microchips with integrated gold electrodes. The layout of the microchips was shown in Fig. 4.5. By increasing the high voltage the current increased linearly up to 300 V. At higher voltages the current dropped to lower values due to the bubble formation at the HV electrodes. Presence of the high voltage electrode in close contact with the separation channel, the products of electrolytic decomposition of water can be used as a source of H^+ and OH^-. At the anode surface the oxidation of water occurs and leads to the formation of H^+ and O_2 gas (equation 5.1) while at the cathode surface the reduction of water takes place leads to the formation of H_2 and OH^- (equation 5.2) (Macounova et al. 2000).

At the anode $H_2O \rightarrow 2H^+ + 1/2O_2 + 2e^-$ (5.1)

At the cathode $H_2O + 2e^- \rightarrow 2OH^- + 2H_2$ (5.2)

The formation of H^+ and O_2 gas on the HV electrode cut the electrical contact in the reservoirs or in the separation channel. Thus, the maximum high voltage which was applicable without bubbles formation was + 300 V. Data are shown in Fig. 5.59 and resistance obtained from linear regression is given in Table 5.15.

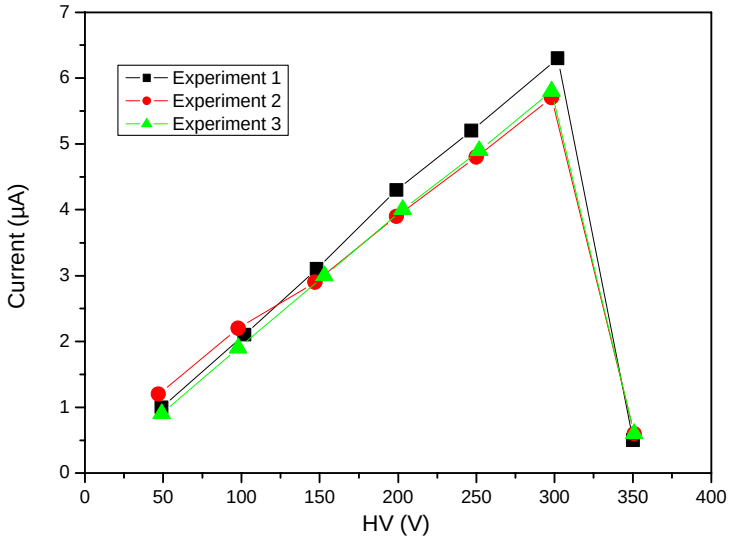


Fig. 5.59 The relation between the applied electrical fields and the resulted currents for ceramic microchips with integrated electrodes.

	1	2	3	Mean
Resistance (MΩ)	47.29	50.9	56.85	51.68
SD	2.22	1.697	3.833	2.583
R	0.999	0.999	0.999	0.999

Table 5.15 The resistance and statistical data for ceramic chips with integrated gold electrodes.

5.2.2.3.2 Electrochemistry of H_2O_2 at the integrated gold electrode

The oxidation of H_2O_2 on integrated gold electrodes was studied using cyclic voltammetry, where H_2O_2 showed an oxidation peak at + 700 mV (*vs.* gold reference electrode) (Fig. 5.60). Thus, this potential was used in the following experiments for H_2O_2 detection.

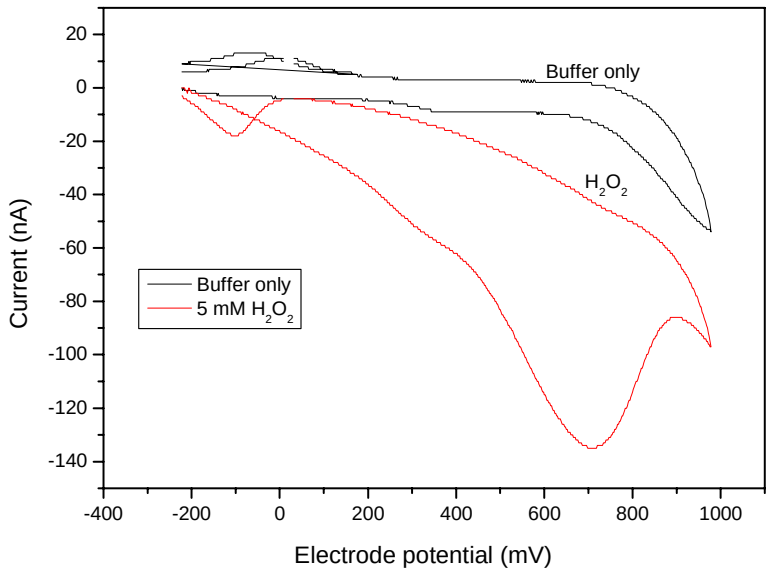


Fig. 5.60 Cyclic voltammogram for 5 mM H_2O_2 at gold electrodes integrated in ceramic microchips (phosphate buffer 10 mM pH 7.3).

5.2.2.3.3 EOF determination

The EOF was measured as mentioned previously (chapter 4.3.6.2) using phosphate buffer 7.3 and H_2O_2 as a neutral marker. Using 300 and 150 V high voltage, the obtained EOF was 19.6 and 13.519 $\pm$ 0.238 mm/min, respectively, and the obtained electroosmotic mobilities were 3.201 and 4.416 mm^2/V min, respectively.

5.2.2.3.4 Effect of high voltage on the signals

High voltage has a great effect on the electrochemical detection systems used in microchips, which depends on the position of the detector with respect to the separation channel. The effect of high voltage on the signals obtained for H_2O_2 and ascorbic acid was examined using different electrode potentials. As shown in Fig. 5.61 the signals for H_2O_2 were decreased by ~ 90 to 50% by increasing the applied potential from 600 to 1200 mV by application of + 300 V. Also the ascorbic acid signal was decreased by ~ 60 to 30% due to the application of 300V. This behaviour is probably due to the position of the gold working electrode. As seen from Fig. 5.62 the working electrode is not directly placed at the end of the separation channel, which led to increase the effects from high voltage. Presence of the working electrode at the end on the separation channel led to direct drop for the applied high voltage at the end of the separation channel, so the working electrode become isolated from the high voltage (Huang et al. 1991; Lu and Cassidy 1994).

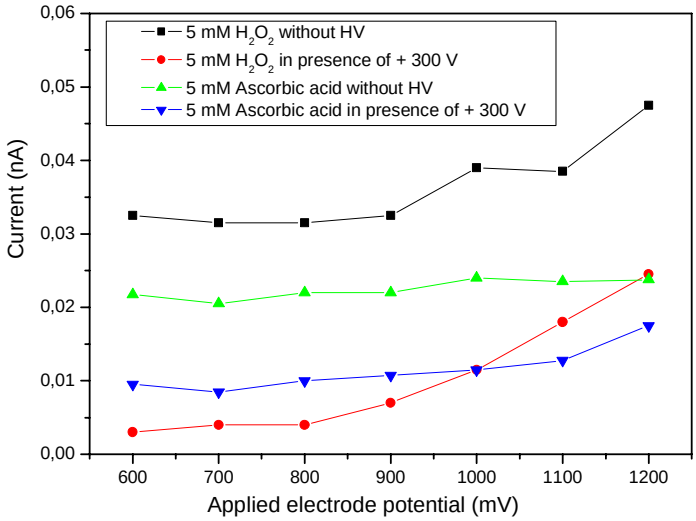


Fig. 5.61 The effect of high voltage on the response for H_2O_2 and ascorbic acid at gold integrated electrodes in ceramic microchips.

5.2.2.3.5 Separation of H_2O_2 and ascorbic acid

The basic material in the ceramic microchips fabrication is aluminum borosilicate, which should generate an electroosmotic flow similar to that of fused silica (Henry et al. 1999). The migration of neutral H_2O_2 and negatively charged ascorbic acid was investigated. Despite the effect of the high voltage on the response for both H_2O_2 and ascorbic acid, the two compounds were separated and detected using the integrated gold electrodes. A mixture of 10 mM H_2O_2 and 50 mM ascorbic acid was injected to the BR using + 300 V injection voltage for 30 s. The same voltage was applied to the separation channel between the BR and BW and the resultant diagram is shown in Fig. 5.63. H_2O_2 was transported faster than the negatively charged ascorbic acid.

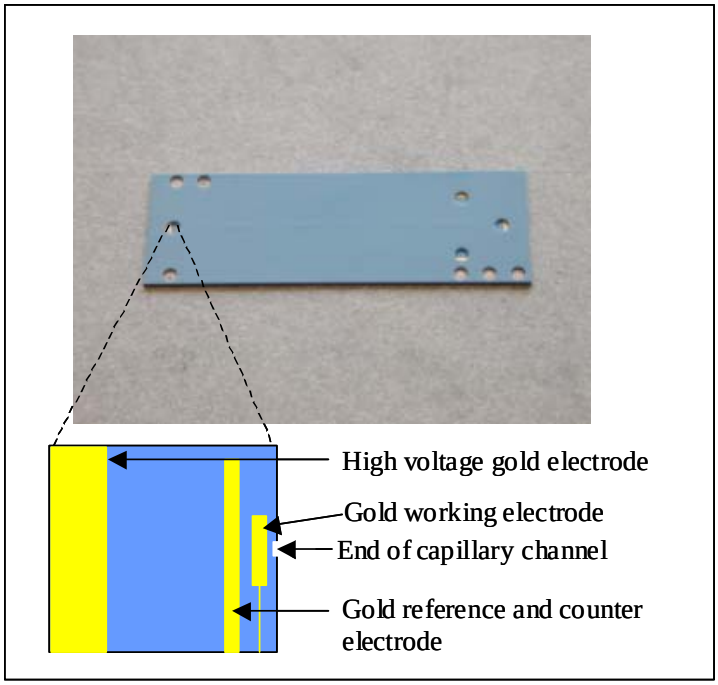


Fig. 5.62 Ceramic microchips with integrated electrodes and the position of working, reference, counter and high voltage electrodes.

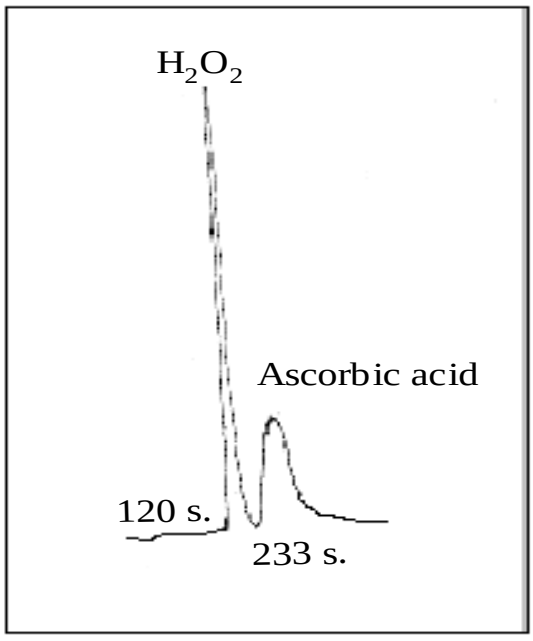


Fig. 5.63 Separation of 10 mM H_2O_2 and 50 mM ascorbic acid using ceramic microchips with integrated gold electrodes.

5.2.2.3.6 Separation of dopamine and catechol

Dopamine and catechol can also be detected by amperometric detection using the integrated gold electrodes. Dopamine as a positively charged molecule was transported faster than catechol (Fig. 5.64).

Thus, ceramic microchips could be used for the detection of neutral, negatively and positively charged compounds. The transportation of these compounds was accomplished by the generated EOF. However broad peaks were obtained which decreased both the sensitivity and the selectivity in this kind of microchips. The peak broadening may be due to the position of the working electrode with respect to the separation channel (Fig. 5.62). This peak broadening was observed before in ceramic microchips with manual placement of electrodes (Henry et al. 1999).

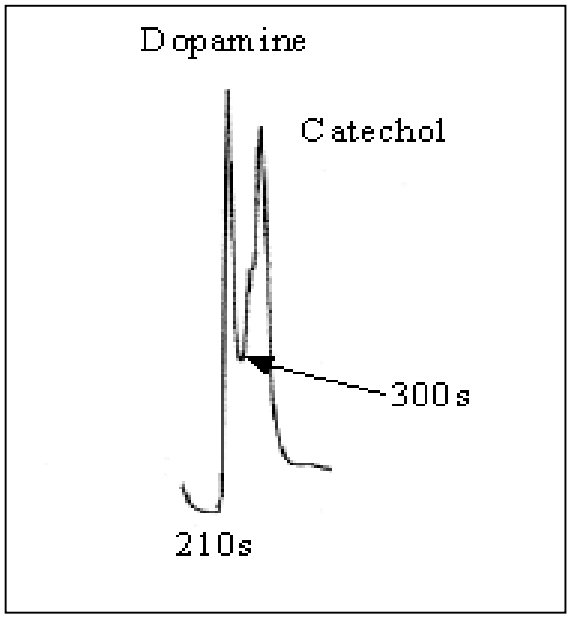


Fig. 5.64 Separation of 1 mM dopamine and 3 mM catechol using ceramic microchips with integrated gold electrodes using phosphate buffer 10 mM (pH 6.85), + 300 V injection voltage for 20 s, + 300 V separation voltage and 600 mV electrode potential.

5.2.2.4 Comparison

Chip Material	Maximum high voltage possible (V)	Maximum current obtained (µA)	Resistance (MΩ)	High voltage for EOF (V)	Electroosmotic mobility (mm^2/V.min)
Glass	6000	37.3	205	2000	1.353
PMMA	6000	22	480.2-543.7	2000	0.7905
Ceramic	300	10.3	51.68	300	3.201

Glass microchips were taken as a reference material. Capillaries in PMMA and ceramic chips showed a lower conductivity than capillaries in glass microchips under comparable experimental conditions. Comparison between the different types of microchips are collected in Table 5.16.

Table 5.16 Comparison between the different properties for glass, PMMA and ceramic microchips.

5.2.3 Determination of glucose using soluble glucose oxidase

In the previous work on the determination of glucose using enzyme electrodes, the interferences of glucose with other electrochemical compounds, which oxidized at the same electrode potential for H_2O_2 as the product from the enzymatic reaction between glucose and glucose oxidase had been investigated. The influence of different additives to reduce or eliminate these interferences was tested. Capillary electrophoresis is a powerful technique for separation for many biological compounds, therefore it may be a good strategy to eliminate interferences.

Determination of glucose with soluble GOD in the running buffer using capillary electrophoresis in glass microchips was mentioned by Wang et al. (2000a). In this work, 75 U/mL GOD was used in the running buffer, while higher GOD concentrations showed adsorption of the enzyme onto the channel surface. As mentioned previously (chapter 2.2.2.2.2) the adsorption of enzyme or generally protein onto the inner surface reduced the sensitivity and selectivity of this system due to changing in the EOF. To eliminate the adsorption of enzyme onto the capillary surface one possible approach was coating the capillary surface using different types of surfactants. Addition of surfactants has three main effects:

1- Dynamic coating by the interaction with the surface leads to changes of the magnitude and the polarity of the EOF.
2- Below CMC, surfactants interact selectivity with analytes and background (BGE) electrolyte components, which changes the EOF.
3- Properties of the BGE are changed such as viscosity.

We investigated the influence of different surfactants, in particular negatively or positively charged surfactants, using three different microchips materials (glass, PMMA and ceramic), which may be eliminate enzyme adsorption.

5.2.3.1 Influence of positively charged surfactants on the EOF

5.2.3.1.1 CTAB

The migration behavior of analytes depend on the electroosmotic and the electrophoretic mobilites of analytes. The influence of the single-chained cationic surfactant cetyltrimethylammonium bromide (CTAB) on the migration time for H_2O_2 in glass, PMMA and ceramic microchips was investigated.

5.2.3.1.1.1 Glass microchips

The CMC value for CTAB (Fig. 5.65) is 0.15 mM in phosphate buffer pH 9.0 (Lucy and Underhill 1996). The effect of CTAB on the EOF was studied from 0.05 to 0.8 mM at three different pH values, 3.5, 7.2 and 9.0. In low concentrations, CTAB is present as a single chained form, while at concentrations above the CMC it forms spherical micelles in solutions (Fig. 5.66a). The mechanism of surfactants adsorption on silica surface was depicted as a bilayer (Melanson et al. 2000). It was assumed that a monolayer is initially formed into which tail groups from a second layer become intertwined, resulting in a flat bilayer structure (Fig. 5.67). Using atomic force microscopy (AFM), Liu and Ducker (1999) indicated spherical aggregates of CTAB at silica surface (Fig. 5.68).

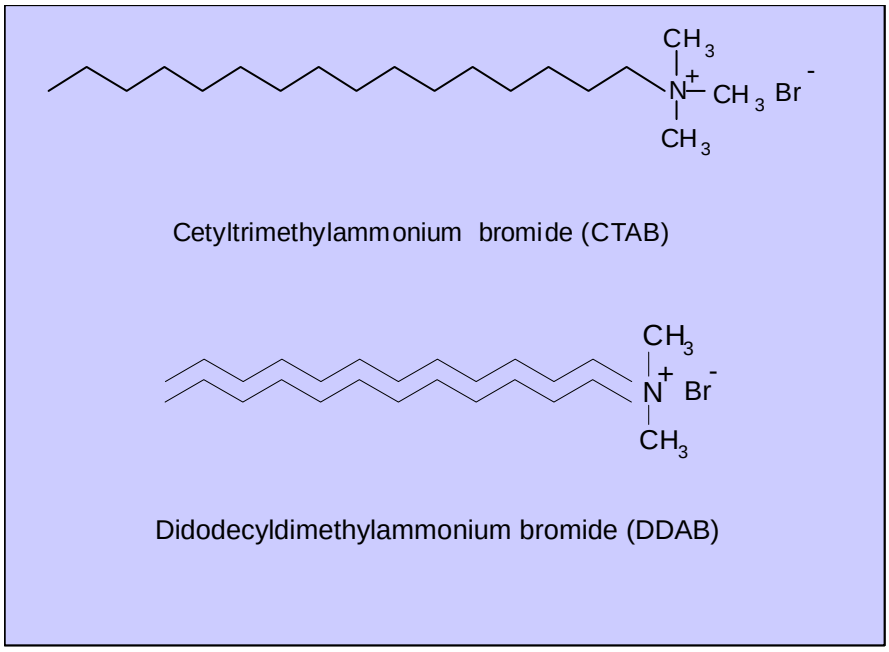


Fig. 5.65 The chemical structure for CTAB and DDAB.

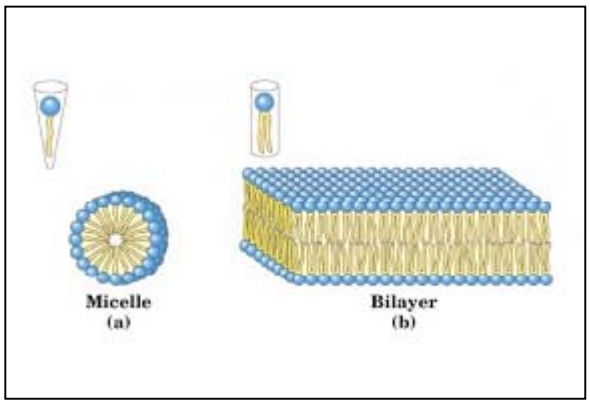


Fig. 5.66 Aggregate structure of (a) single-chained surfactants and (b) double-chained surfactants.

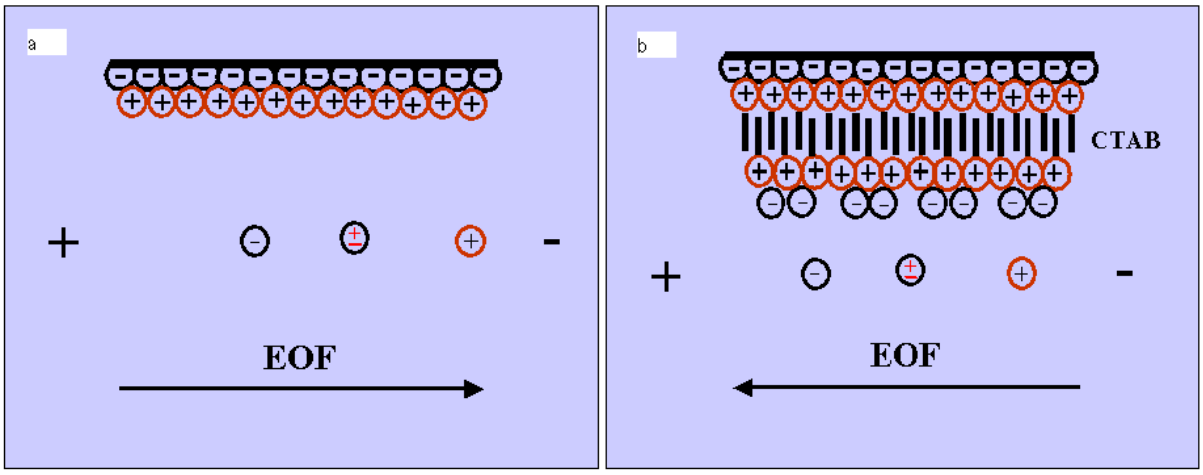


Fig. 5.67 Glass surface in absence (a) and presence (b) of the single-chained CTAB.

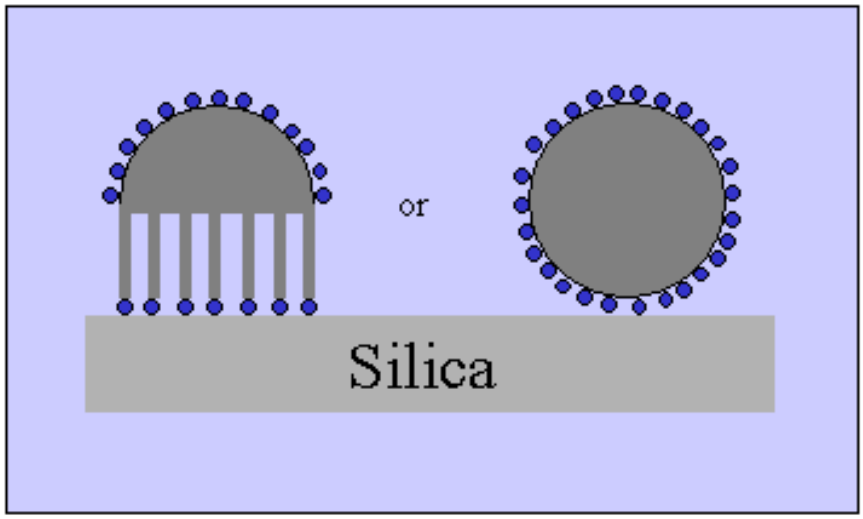


Fig. 5.68 CTAB forms half micelles on a flat monolyer or a full micelle on silica surface.

The influence of different concentrations of the single-chained cationic surfactant CTAB below and above its critical micelle concentration (CMC) on the electroosmotic mobility was investigated. Addition of low CTAB concentrations till 0.01 mM reduced the EOF (0.01 mM CTAB reduced the EOF by ~ 54%) (Fig. 5.69). For a concentration of about 0.05 mM CTAB the EOF was almost zero. For concentrations higher than 0.05 mM, the EOF was reversed to the anodic direction and showed an increase in this direction up to 0.3 mM CTAB. Higher concentrations led to no further increase (Fig 5.69). Probably the inner surface of the microchips was completely coated by CTAB and reached a saturation state.

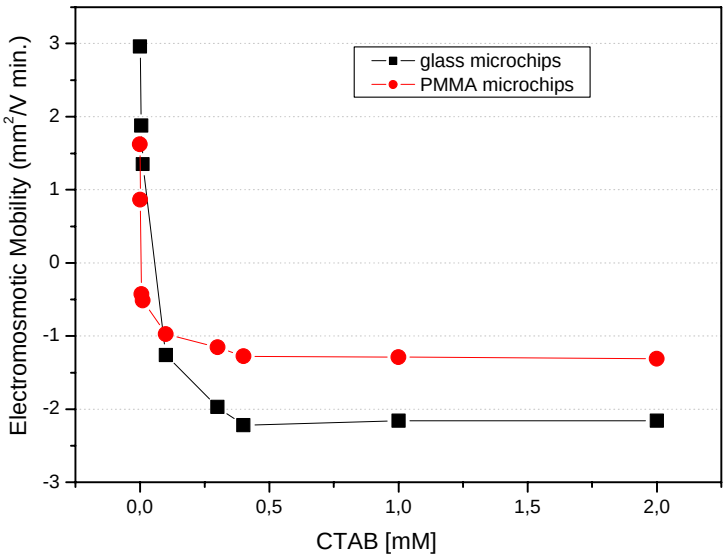


Fig 5.69 Effect of CTAB on the electroosmotic mobility in glass and PMMA microchips using 1 mM H_2O_2 as neutral marker.

5.2.3.1.1.2 Poly(methylmethacrylate) (PMMA) microchips

Addition of 0.001 mM CTAB reduced the EOF by about 38%, while with concentrations higher than 0.005 mM the EOF was reversed towards the anode (Fig. 5.69). The dynamic coating of PMMA microchips was more effective than of glass microchips. In glass microchips probably the ionized silanol groups interacted with the positively charged head groups of CTAB, whereas in PMMA chips the hydrophobic part of the surfactant molecule can interact with the hydrophobic surface of the polymer (Fig. 5.70). Thus, in glass microchips the reduction of the EOF may be due to a compensation of negative charges by ionic interactions, whereas on PMMA surfaces additionally positive charges are introduced.

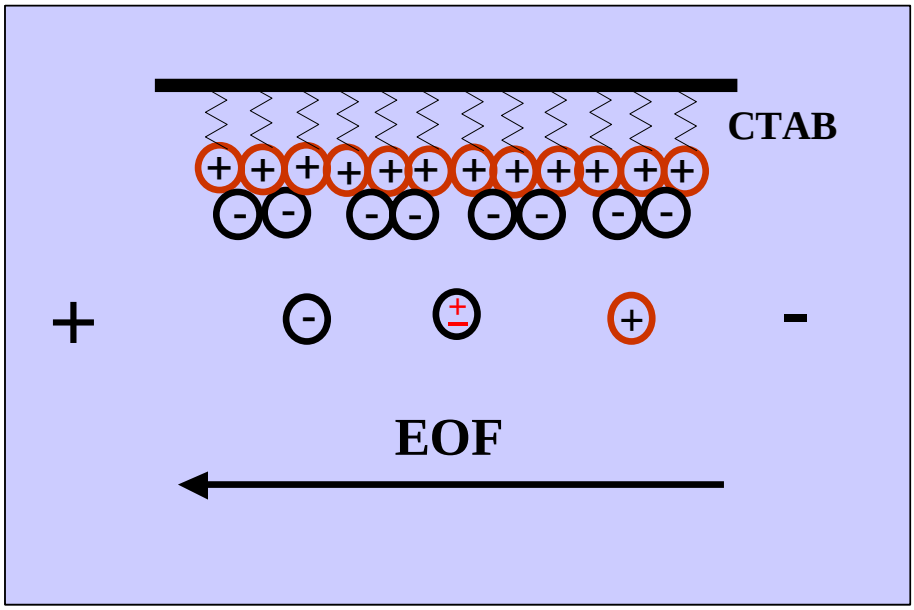


Fig. 5.70 Adsorption of CTAB on the PMMA polymer surface.

5.2.3.1.1.3 Ceramic microchips

The effect of CTAB on the EOF in ceramic microchips is shown in Fig. 5.71. Increasing the CTAB concentrations decreased the electroosmotic mobility up CTAB concentrations of to 0.3 mM, at higher concentrations the EOF was reversed to the anodic direction. Thus, in ceramic chips the reversal of the EOF was observed at rather high CTAB concentrations of 0.4 mM. This behaviour may be due to the porosity of ceramic surface, which may be higher than of both glass and PMMA chips. Thus, increased inner surface area and higher concentrations from the surfactant were needed to charge the surface.

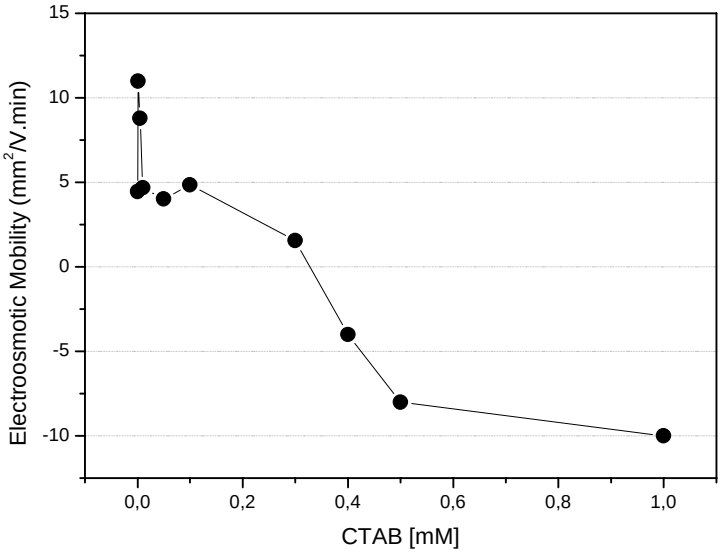


Fig. 5.71 Effect of CTAB on the electroosmotic mobility in ceramic microchips.

5.2.3.1.2 Double-chained cationic surfactant DDAB

The double-chained cationic surfactant DDAB (Fig. 5.65) forms stable coatings onto the wall of capillaries even after washing the channel with a surfactant-free solution for 75 min (Melanson et al. 2000). This was not observed for the single-chained surfactant CTAB and other surfactants, which must be present in the running buffer during experiments. The double-chained surfactant form aggregates at concentrations above a critical vesicle concentration (CVC). For DDAB the CVC in water at 25 ^{o}C is 0.035 mM (Svitova et al. 1995).

The influence of various concentrations from the double-chained surfactant DDAB on the migration time for 1 mM H_2O_2 in phosphate buffer (10 mM, pH 7.3) was examined. Different protocols were used for coating microchips by DDAB:

1- DDAB was dissolved in phosphate buffer pH 7.3 and the coating process was accomplished by rinsing the glass microchip channels with the surfactant solution for 10 min followed by washing with phosphate buffer (10 mM, pH 7.3) for 3 min which was also used as a running buffer (Melanson et al. 2000).
2- Microchip channels were rinsed by DDAB (in phosphate buffer) for 30 min followed by washing with phosphate buffer for 3 min.
3- Microchip channels were rinsed with DDAB (in phosphate buffer) for 30 min and this solution was also used as a running buffer.

The resultant effects on glass chips using the three different protocols are shown in Fig. 5.72.

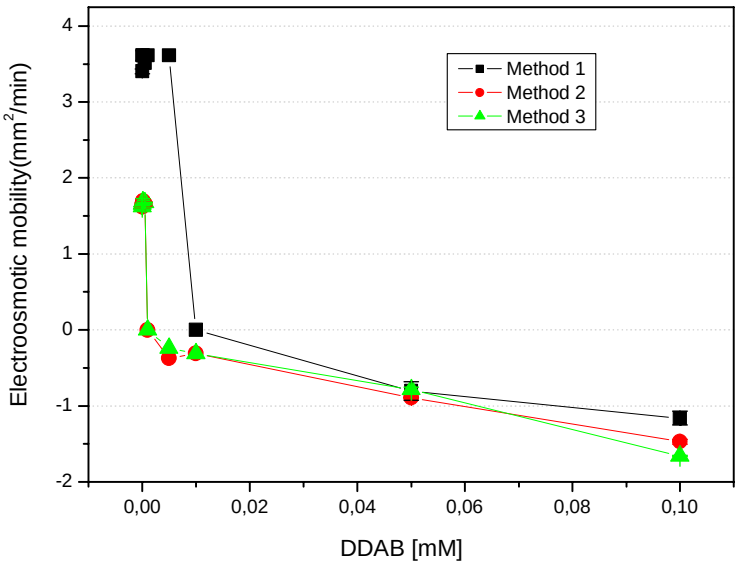


Fig. 5.72 Effect of the double-chained surfactant DDAB on the electroosmotic mobility in glass microchips, DDAB in 10 mM phosphate buffer pH 7.3.

When the channels were coated by method 1, low concentrations of DDAB (up to 0.01 mM) did not show any marked effect on the migration time for H_2O_2. Thus no reduction of the EOF was observed. For higher DDAB concentrations, above the CVC (0.05 mM), the EOF was reversed. The second and third protocol delivered almost identical results, which, however, were different from the first. At low DDAB concentrations, up to 0.001 mM, there was no effect for DDAB on the EOF, as there was no change in the migration time for H_2O_2. At 0.005 mM DDAB the EOF was almost zero. At higher concentrations the EOF was reversed to the anodic direction.

Baryla and Lucy (2002) reported a method for capillary coating by DDAB, which included rinsing the capillary with 0.1 mM of DDAB in 20 mM Tris-HCl buffer containing 20 mM $CaCl_2$ (pH 7.4) for 5 min. They found that addition of $CaCl_2$ promoted the coating, but did not explain this effect. Accordingly, the influence of $CaCl_2$ was also studied using different DDAB concentrations in 20 mM Tris-HCl (pH 7.4). Glass chips were coated by rinsing the capillary with the DDAB-Ca^{2+}-solution for 5 or 30 min followed by washing with phosphate buffer (pH 7.3) for 3 min. As shown in Fig. 5.73, with DDAB concentrations up to 0.01 mM no effect on the EOF was observed, irrespective of the coating time. Higher concentrations led to a reversal of the EOF. Thus, coating of glass microchip channels by DDAB in different buffers (phosphate or Tris-HCl + $CaCl_2$) gave the same results. The addition of DDAB did not show a gradual reduction for the EOF, but a rapid transition from normal to reversed EOF was observed. This behaviour was recently also reported by Lucy and Diress (2004) for fused

silica capillaries, where a rapid transition from normal to reversed EOF was observed as the DDAB concentration increased from 0.05 to 0.1 mM.

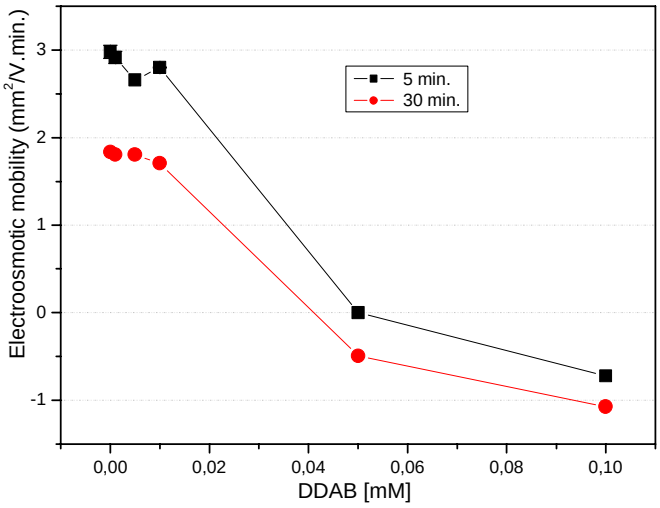


Fig. 5.73 The effect of double-chained surfactant DDAB on the electroosmotic mobility in glass microchips, DDAB in 20 mM Tris-HCl buffer containing 20 mM $CaCl_2$ (pH 7.4).

The effect of the coating by different DDAB concentrations (for 30 min) on the signal for 1 mM H_2O_2 was also examined. At low DDAB concentrations where the migrations occurred by the normal EOF no marked effect on the response for H_2O_2 was observed. At higher DDAB concentrations, above the CVC with a reversed EOF, the signals for H_2O_2 dropped to values more than 80% lower than the value without coating or with normal EOF (Fig. 5.74). This drop in the response for H_2O_2 was not observed when CTAB was used as surfactant.

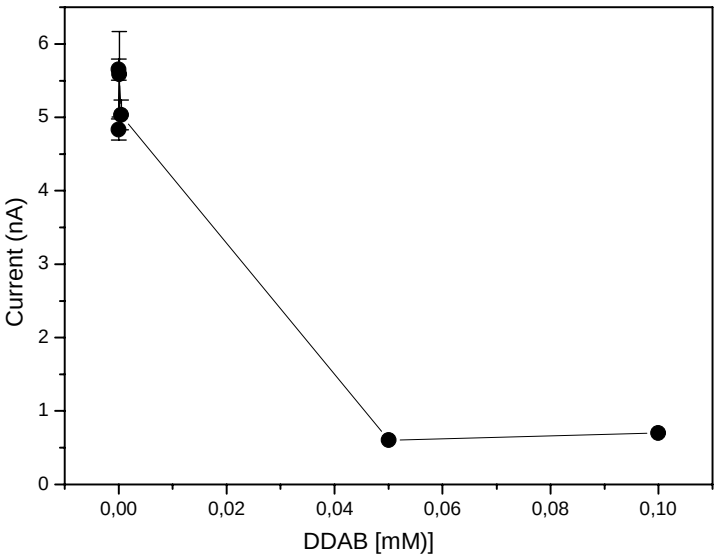


Fig. 5.74 Influence of DDAB coating on the signals for 1 mM H_2O_2.

5.2.3.2 Influence of the negatively charged surfactant sodium dodecylsulfate (SDS)

SDS (Fig. 5.75) is an anionic surfactant with a negatively charged sulfonate group and a hydrophobic carbon chain. SDS is the most commonly surfactant in MEKC and was extensively used in the separation of proteins by capillary electrophoresis, where it enhances the EOF by increasing the density of negative charges on the inner capillary surface. At low concentrations (< 1 mM) in water, individual SDS molecules exist as single entities. By increasing SDS concentration to 8 mM in water, the individual molecules begin to form molecular aggregates which known as the CMC. As mentioned above, the CMC for SDS in water is approximately 8 mM, although this value will vary when salts, organic solvents, etc., are added to the solution (Landers 1997). The influence of SDS on the EOF in different microchip materials was investigated.

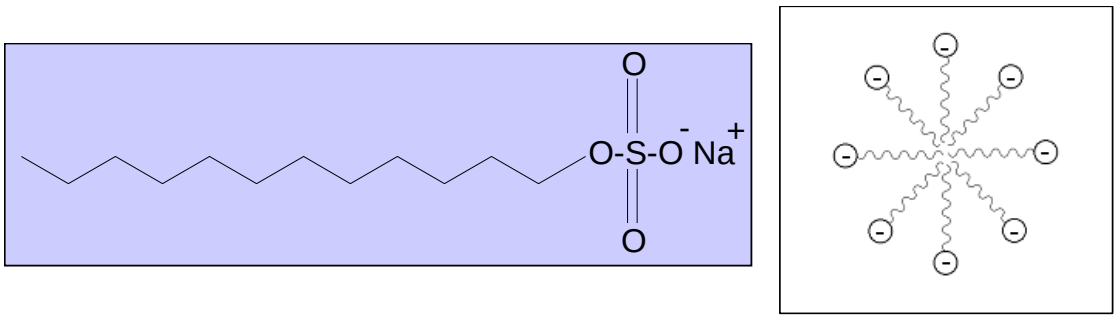


Fig. 5.75 Molecular structure for the anionic surfactants SDS (a) and the SDS micelle (b).

5.2.3.2.1.1 Glass microchips

The influence of the SDS concentration on the EOF in glass microchips was investigated. Adsorption of the anionic surfactant SDS enhanced the EOF because it increased the negative charge density on the inner surface of the capillary (Fig. 5.76). SDS concentrations below 0.01 mM showed only a small effect on the EOF, while addition of 0.05 and 0.1 mM SDS increased the EOF from 31.8 mm/min (without SDS) to 46.4 and 51.5 mm/min, respectively. At higher SDS concentrations only small further increases of the EOF were observed (saturation).

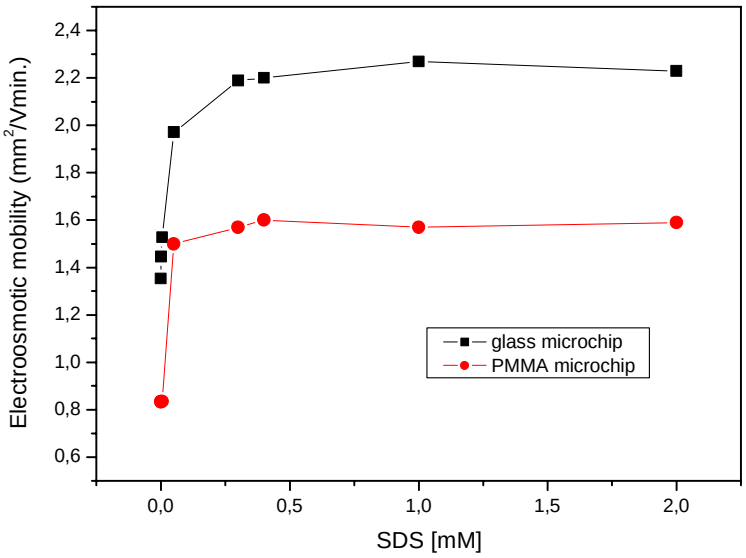


Fig. 5.76 Effect of SDS on the electroosmotic mobility in glass and PMMA microchips.

5.2.3.2.1.2 PMMA microchips

The effect of SDS on the EOF in the PMMA microchips was more pronounced (Fig. 5.76), as the addition of only 0.05 mM SDS increased the EOF from 19.6 mm/min (without SDS) to 36.5 mm/min. Higher SDS concentrations showed only a very small effect on the EOF. Obviously, the surface was saturated with SDS at a SDS concentration, which was lower for the PMMA microchips than for the glass microchips, probably due to the higher hydrophobicity of the polymer.

5.2.3.2.1.3 Ceramic microchips

The influence of SDS on the EOF of ceramic microchips showed a completely different behaviour, as the EOF decreased with increasing SDS concentrations and this decrease occurred in the narrow concentration range up to 0.2 mM SDS (Fig. 5.77). At higher SDS

concentrations the EOF decreased only gradually. There is no explanation for this strange behaviour till now.

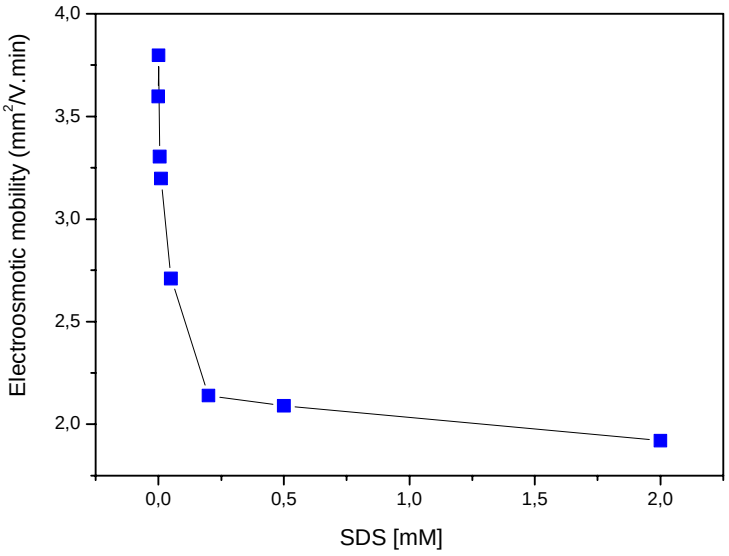


Fig. 5.77 Effect of SDS on the electroosmotic mobility in ceramic microchips.

5.2.3.2.1.4 Summary

CTAB showed different coating behaviours in the different types of microchips. The effect of CTAB on PMMA chips was higher than in glass and ceramic microchips, as the EOF was reversed after surface coating using concentrations higher than 0.005 mM while concentrations higher than 0.05 and 0.4 mM were required for glass and ceramic chips, respectively. In PMMA channels the adsorption mechanism for surfactants are probably hydrophobic-hydrophobic interactions, while for ceramic the electrostatic interaction is the predominant interaction. Thus, at low CTAB concentrations positive and negative charges compensate each other, so that higher concentrations may be needed to charge the surface. Coating glass by DDAB showed another behaviour, as no gradual decrease for the EOF was observed by increasing DDAB concentrations but a rapid transition from the normal to the reversed EOF was observed. Coating of microchips with SDS was also examined, where in case of glass and PMMA an enhancement for the EOF was obtained and it was higher in case of PMMA than glass. For example addition of 0.05 mM from SDS to the buffer, the EOF increased from 31.8 to 46.4 in case of glass chips and from 19.6 to 36.5 in case of PMMA chips. But a different behaviour was observed in case of ceramic microchips where by increasing SDS concentrations the EOF decreased. The maximum electroosmotic mobilities for the different types of microchips in the absence and presence of SDS and CTAB are shown in Table 5.17.

Chip material	Maximum mobility (mm^2/V.min)			
	without	SDS	CTAB	DDAB
Glass	1.6	2.2	- 2.1	- 1.47
PMMA	1.2	1.55	- 1.3	not detected
Ceramic	3.55	3.7	- 10.0	not detected

Table 5.17 Maximum mobilities obtained using different microchip materials in the absence and in the presence of different surfactants.

5.2.3.3 Determination of glucose

After coating the inner surface of microchips using the cationic and anionic surfactants, the migration times for glucose and ascorbic acid were investigated. Glucose was determined in fruit juices using the optimal conditions in glass and PMMA microchips after coating by the suitable surfactant.

5.2.3.3.1 The influence of surfactants on the separation of H_2O_2 and ascorbic acid

5.2.3.3.1.1 Cationic surfactants

Ascorbic acid was used as a model interferant in all investigations. Usually it is present in its dissociated form (anion), which is repelled electrostatically from the cathode. This led to larger migration times compared to the neutral H_2O_2. The migration time for ascorbic acid in glass microchips was ~ 133 s $\pm$ 2.0 s and for H_2O_2 ~ 73 s $\pm$ 2.0 s. The addition of 0.01 mM CTAB significantly reduced the EOF (without reversing) (see Fig. 5.67) leading to an increase of the migration time of H_2O_2 to ~ 96 s $\pm$ 1.0 s. For ascorbic acid the effect was even more pronounced and a migration time of 253 s $\pm$ 2.0 s was obtained. Thus, the reduction of the EOF improved the separation efficiency for glucose and ascorbic acid. This may be due to additional electrostatic interactions between the cationic surfactant adsorbed on the capillary wall and the anions leading to an additional retention.

Similar effects were observed in the PMMA microchips. The transportation time for H_2O_2 was ~ 82 s $\pm$ 1.0 s while ascorbic acid reached the detector after ~ 209 s $\pm$ 2.0 s. With 0.001 mM CTAB the migration times for H_2O_2 and ascorbic acid increased to ~ 118 s $\pm$ 1.0 s and 1050 s $\pm$ 9 s respectively (Fig.5.78), thus the separation of the two compounds became more effective in presence of lower CTAB concentrations.

The double-chained DDAB did not show a gradual decrease in the EOF at concentrations lower than the CVC, but reversed it at concentrations above the CVC as mentioned before. Reversal of the EOF using higher concentrations of CTAB or DDAB reduced the migration times for ascorbic acid. For example using 0.2 mM CTAB and - 2 kV, ascorbic acid and H_2O_2

were transported for ~ 70 s ± 3.0 s and 79 s ± 2.0 s, respectively, so that the separation efficiency of ascorbic acid from H_2O_2 was reduced.

5.2.3.3.1.2 Anionic surfactant

Adsorption of the anionic surfactant SDS increased the negative charge on the inner surface of the capillary and enhanced the EOF of glass and PMMA chips (Fig. 5.76). By addition of 0.01 mM SDS to a phosphate buffer, the migration times for glucose and ascorbic acid were reduced from 82 s ± 1.0 s and 153 s± 3.0 s to 65 s ± 1.0 s and 104 s ± 1.0 s respectively. The presence of SDS enhanced the EOF and this had a stronger effect on the negatively charged ascorbic acid than on the neutral molecule H_2O_2.

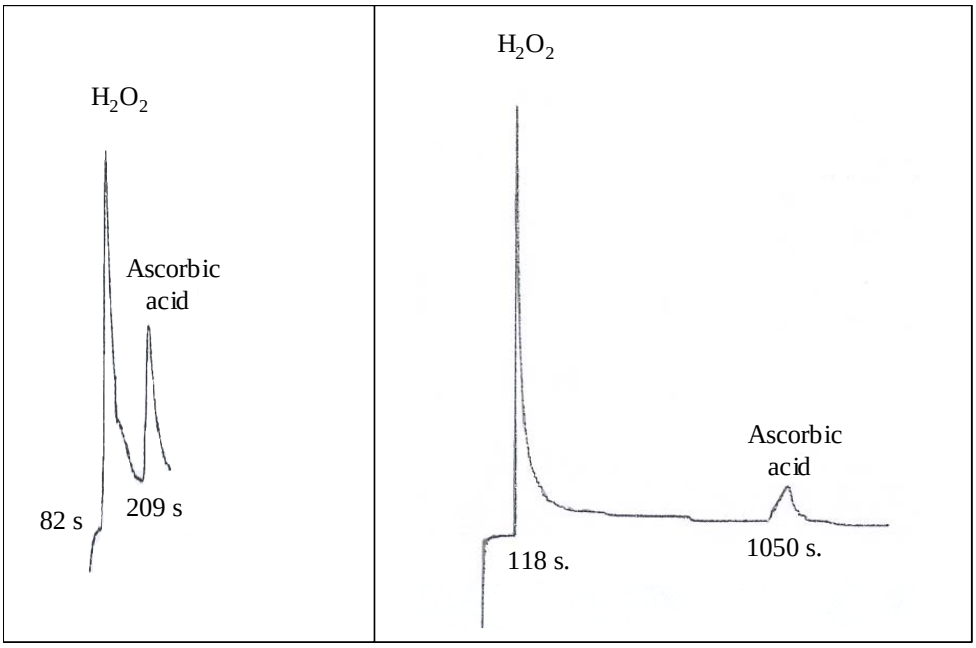


Fig. 5.78 Effect of 0.001 mM CTAB on the migration times for 1 mM H_2O_2 and 4 mM ascorbic acid in PMMA chips.

5.2.3.3.2 Effect of separation voltage on the migration times for H_2O_2 and ascorbic acid

As the EOF is dependent on the applied electric field strength, the transportation voltage has a great effect on the migration times and in turn the separation is improved or deteriorated. Fig. 5.79 shows the influence of the transportation voltage on the migration times for H_2O_2 (produced from enzymatic oxidation of glucose) and ascorbic acid using 0.01 mM CTAB as buffer additive. By increasing the transportation voltage from + 500 to + 3000 V, the migration times decreased. This effect was more pronounced for ascorbic acid than for H_2O_2 in the voltage range from 500 to 2000 V, reducing the differences in migration times from almost 500 s (using 500 V) to 120-150 s (using 2000 V). A further increase did not lead to

further significant changes. As the difference in migration time of 120-150 s was sufficient, 2 kV were applied in following experiments.

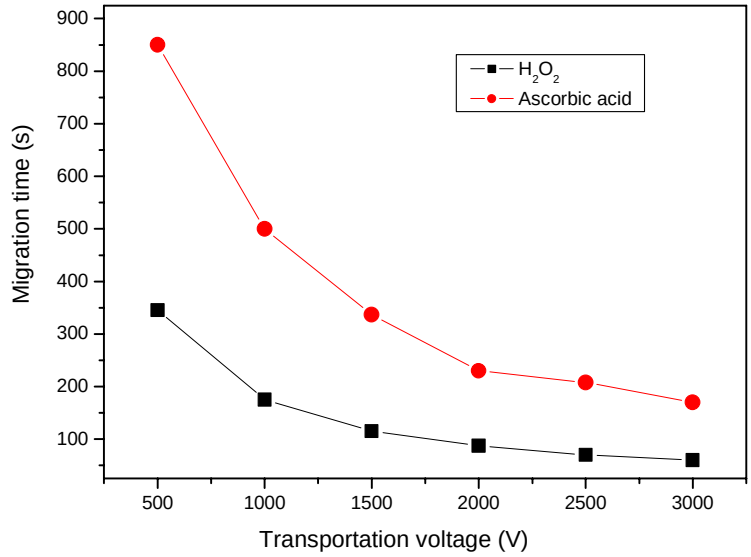


Fig. 5.79 The effect of transportation voltage on the migration times for H_2O_2 and ascorbic acid. Mixture of 0.5 mM H_2O_2 and 0.5 mM ascorbic acid in phosphate buffer (10 mM, pH 7.3) + 0.01 mM CTAB, + 700 mV electrode potential, 2 kV injection voltage for 10 s.

5.2.3.3.3 Optimization of electrophoretic conditions for glucose determination

The sample injection mode in microchip capillary electrophoresis has a great effect on the shape of the obtained peaks. 3 different injection modes are described: pinched, unpinched and gated injection (see chapt. 2.2.2.4.2.3).

For the injections of samples in microchips, the unpinched injection was investigated by two methods. In the first method the sample was injected by application of high voltage to the SR with the BW grounded while the BR and SW were floating. In second method the sample was injected by application of the high voltage to the SR and grounded the SW and the BR and BW were floated. In both cases the sample transportation was accomplished by the application of the high voltage to the BR and the 85 % of this value was applied to SB and SW, with BW was grounded. The shape and the height of the obtained peaks were different, where in the first mode higher and sharper peaks were obtained (Fig. 5.80).

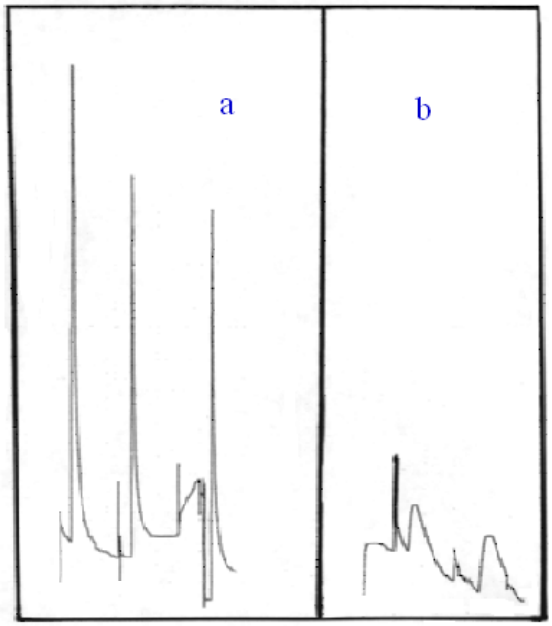


Fig. 5.80 (a) Injection from SR to BW (b) Injection from SR to SW.

5.2.3.3.4 Determination of glucose in glass microchips.

Different concentrations of glucose oxidase ranged from 10 to 100 U/mL were added to the running buffer and the signals for 0.5 mM glucose solution were measured. By increasing the enzyme concentration, glucose signals increased up to 100 U/mL. Higher glucose oxidase concentrations were not examined and 100 U/mL GOD was used for further investigations.

Using 100 U/mL GOD in the running buffer, different glucose concentrations from 0.1 to 1.0 mM were used. By increasing glucose concentrations the signals increased, leading to a linear range from 0.1 to 0.9 mM (Fig. 5.81) with a correlation a coefficient R = 0.998 and standard deviation of 3.381.

Using the optimal conditions mentioned before, glucose was determined in three different types of juices (No. 5, 6 and 7 in Table 5.1) diluted 1:200. The resultant data were compared to data obtained from the reference method (YSI) (Fig. 5.82) and showed a good correlation. The statistical t-tests showed a good relation between the two systems with correlation coefficient of 0.999 and standard deviation of 1.094. The t-value obtained using 0.05 degree of freedom is 0.0919.

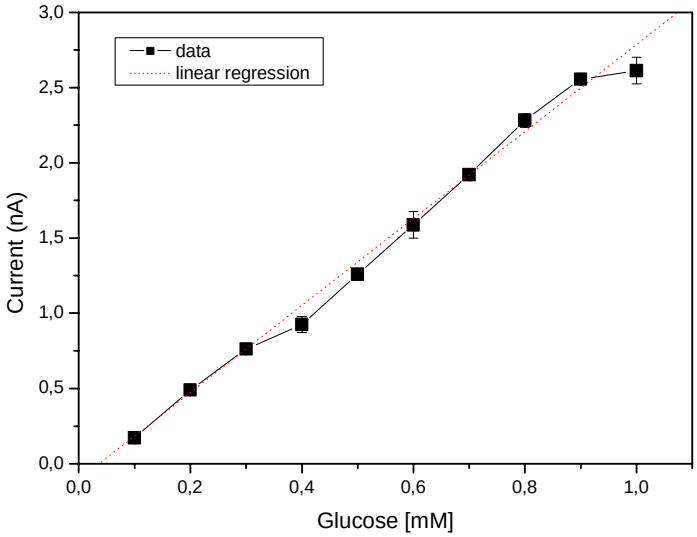


Fig. 5.81 Calibration curve for glucose using glass microchips. 2 kV injection for 10 s.

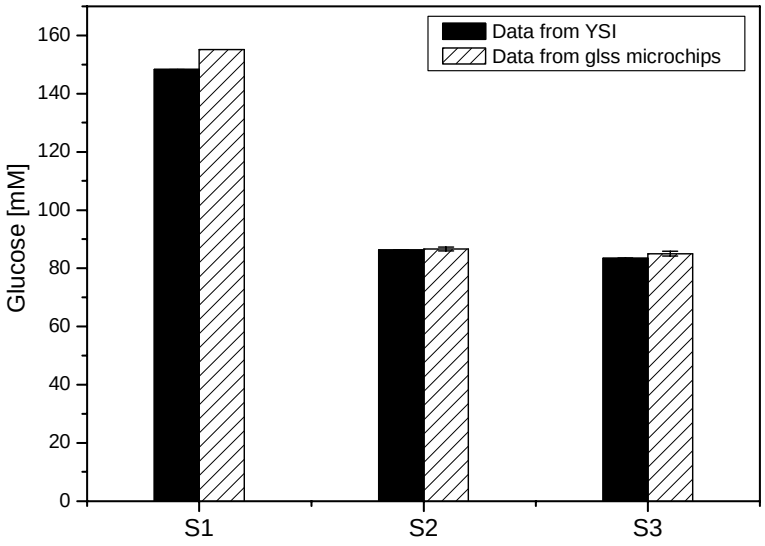


Fig. 5.82 Determination of glucose in three different juice samples using YSI and glass microchips using the same conditions as in the experimental part.

5.2.3.3.5 Determination of glucose in PMMA microchips.

For the determination of glucose in the polymer microchips (PMMA), the same buffer compositions as used for glass microchips were applied except 0.001 mM CTAB was used instead of 0.01 mM CTAB. Different glucose concentrations ranged from 0.05 to 1.0 mM were injected and the resultant response was measured. A linear region from 0.05 to 0.8 mM was obtained with a correlation coefficient of 0.998 and 0.0102 SD (Fig. 5.83).

3 juice samples (No. 5, 6 and 7 in Table 5.1) were diluted 1:200 and the resultant concentrations were compared to those obtained using the standard method (Fig. 5.84). The obtained data from the PMMA microchips showed a good correlation to that obtained from YSI. The statistical tests showed that the two data correlated to each other with R = 0.999, SD = 0.947 and 0.079 t-value using 0.05 degree of freedom.

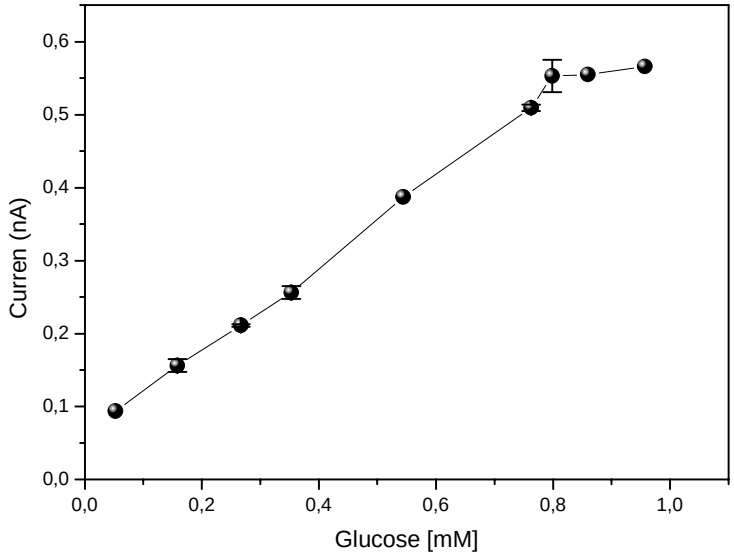


Fig. 5.83 Calibration curve obtained for glucose using PMMA microchips.

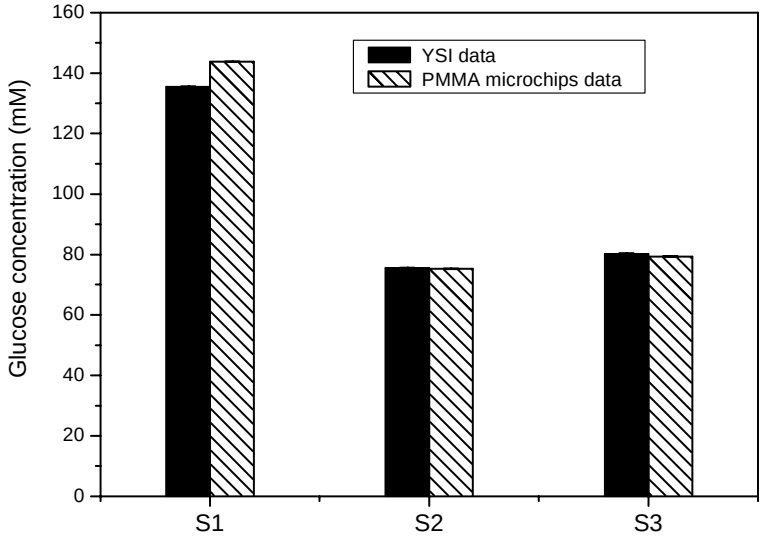


Fig. 5.84 Glucose concentrations in juice samples obtained from PMMA microchips and YSI.

5.2.3.3.6 Determination of glucose in ceramic microchips

To study the effect of surfactants on the transportation time for H_2O_2 in ceramic chips, firstly 1 mM H_2O_2 was transported in phosphate buffer pH 7.3. The transportation time was 109 s $\pm$ 2 s using 100 V injection voltage for 10 s from SR to SW and 300 V transportation voltage. Determination of glucose using soluble enzyme was studied in ceramic chips using phosphate buffer 7.3 and GOD (60 U/mL). The transportation times increased with repeating the experiments i.e. 0.7 mM glucose was detected after 480 s and this value reached 540 s after 3 times repeatation. Thus, the presence of enzyme in the channels decreased the EOF with time, which may be due to the continuous adsorption of enzyme on the inner surface. The effect of 0.1 mM CTAB in phosphate buffer on the transportation time for H_2O_2 was also examined after rinsing the ceramic surface by 0.1 mM CTAB in phosphate buffer for 30 min. H_2O_2 was transported after 216 s $\pm$ 0.6 s. Determination of glucose using soluble enzyme was again examined using 0.1 mM CTAB in phosphate buffer in the presence of GOD 60 U/mL. 1 mM glucose was detected after 212 s and this value reached 261 s after repeating the experiment for 3 times . Thus, the addition of CTAB reduced the EOF in ceramic microchips and not completely reduce the effects due to the presence of enzyme. This again may be due to the porosity of the surface, which may increase the possibility for the interaction with the enzyme. This increased the positive charge on the inner surface, which lead to decrease in the EOF. Thus, for ceramic microchips the migration time continuously increased by repeating the experiment in the presence of enzyme soluble in the running buffer, which led to loss the reproducibility and sensitivity for the system. For this reason no further applications were done in this kind of microchips.

5.2.4 Immobilization of enzyme in microchips

Enzymatic assays were conducted in microchips either by using the enzyme as buffer additives or by enzyme immobilization (Wang 2002; Wang et al. 2004; Peterson et al. 2002). Using enzymes as buffer additives to the running buffer is the most used protocol in enzymatic analysis. However, as mentioned before and shown in the previous chapter, this procedure may suffer from the adsorption of enzyme to the inner wall of the capillary. Thus, immobilization of enzyme in a certain part of the channel system was to be developed to eliminate the problem of adsorption. Also immobilization of enzyme in capillaries reduced the costs due to using soluble enzyme. Due to the high costs of microchips, preliminary experiments were done in fused-silica capillaries. To immobilize enzymes in capillaries to be used for capillary electrophoresis, a suitable protocol had to be developed.

The immobilization procedure had to fulfil several requirements:

a. sufficiently high amount of active enzyme has to be immobilized.
b. the stability should be sufficient to allow repetitive use of the chip.
c. the solution has to flow through the immobilization matrix or has to flow over a layer of immobilized enzyme.
d. transportation of liquids by EOF should be possible, i.e. charged functional groups have to be maintained.

5.2.4.1 Entrapment in polymers obtained by irradiation with UV-light

A mixture of the enzyme and a precursor solution polymerizable by UV-light was passed through the capillary and the capillary was exposed to UV-light. Three different UV polymers were investigated to immobilize GOD on the inner wall of a fused-silica capillary.

5.2.4.1.1 SU 8 paste

SU 8 is a negative, epoxy-type, near UV photoresist and designed for photolithographic structuring in micromaching and microelectronics. Immobilization of GOD using SU 8 in fused silica capillary faced some problems. The first problem was the insolubility of SU 8 in aqueous solutions, which made mixing of all components not complete. Moreover the viscosity of SU 8 increased by dilution with enzyme or buffer, which blocked the capillary during filling. Dilution of SU 8 by organic solvent such as acetone or toluene faced the same problem. SU 8 is soluble in the organic solvent γ-Butyrolactone (GBL), but when aqueous solution were added, the viscosity increased and the polymerization efficiency decreased using UV-light. Thus, this approach was not successful.

5.2.4.1.2 Polyurethane

GOD was immobilized using an aromatic polyurethane dispersion by mixing the polymer with GOD (see the experimental part) and passing the mixture through a 5 cm long capillary. 3 cm from one end of the capillary were coated by Al-foil and then the capillary was exposed to UV light for ~ 10 s. The immobilized matrix was defined by the open space from the capillary, which was not covered by the Al-foil. The immobilization was accomplished and glucose was detected using Pt-wire as a working electrode (with + 800 mV electrode potential). Data are given in Table. 5.18.

5.2.4.1.3 Diacrylate

GOD was mixed very well with the diacrylate paste which was also used for enzyme immobilization on screen-printed electrodes. The capillary was filled with this solution and exposed to UV-light after covering part of the capillary (3 cm) with Al-foil. Glucose was detected by measuring the produced H_2O_2. Data are given in Table 5.18, where glucose was detected after 6.3 min. To increase the EOF the effect of the negatively charged polymer dextran sulfate was examined. Addition of 1% dextran sulfate reduced the migration time, but the signal decreased from 100 to 5 nA.

Material used for immobilization	GOD [mg/mL]	Separation Voltage (kV)	Migration time (min)	Signal height (nA)
Polyurethane polymer	100	+ 1.5	2.2	57.5
Diacrylate polymer	100	+ 1.0	6.3	100.0
Diacrylate polymer+ Dextran sulfate 1%	100	+ 1.0	1.5	5.0

Table 5.18 Comparison between the immobilization method of GOD in fused silica capillaries using two different polymers UV-pastes. The obtained signals using 5 mM glucose.

5.2.4.2 Cross-linking by glutaraldehyde

5.2.4.2.1 Immobilization in fused silica capillary

Glutaraldehyde is considered to be the cross linkers most often used for the immobilization of enzymes in biosensors. Immobilization of GOD on the inner wall of fused silica capillaries was accomplished after activation of the capillary surface by γ-Aminopropyltriethoxysilane (APTES) resulting in primary amino groups (chapter 4.3.6.6). Glutaraldehyde reacted with the

primary amino groups yielding aldehyde group, which form an imine linkage with the primary amino group of GOD according the following mechanism (Fig. 5.85).

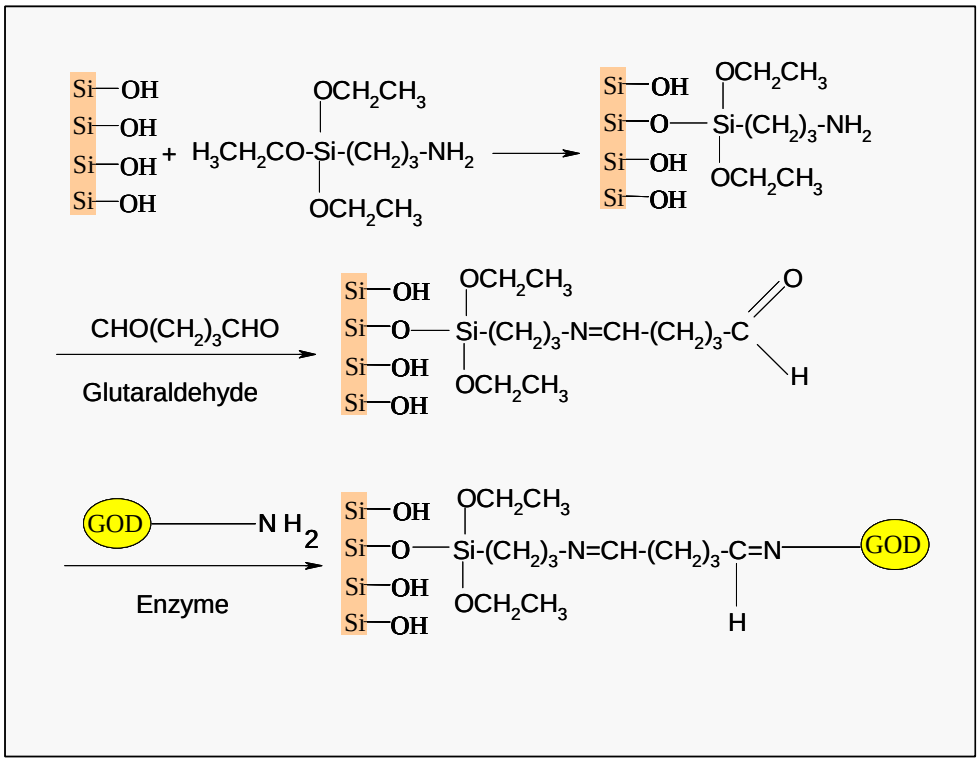


Fig. 5.85 The mechanism for the immobilization of GOD in fused silica capillary using glutaraldehyde as a cross linking agent.

After injection of glucose no signal was obtained even when different high voltages were applied. This was due to the reduction of EOF by replacing the silanol groups from the capillary surface by amino groups from APTES and additionally by the coverage of the surface with enzyme (also see chapter 2.2.2.2.2) Thus, the last step of the immobilization protocol was modified as the enzyme was mixed with 0.25% glutaraldehyde and allowed to react with glutaraldehyde. In this case H_2O_2 was transported and could be detected. The source for the EOF was not the silanol groups present on the capillary surface because the surface was already treated by APTES as shown in Fig. 5.85. No signals for H_2O_2 were obtained after the silanization step. The source of the EOF can be explained by the presence of glutaraldehyde according to the following mechanism. Some glutaraldehyde molecules are oxidized in air to hemi-acid and di-acid derivatives as well as higher oligomers which carry carboxylic groups (Fig. 5.86) (Purss et al. 2003) leading to negatively charged residues in the immobilization matrix and thus allow transportation of compounds by EOF.

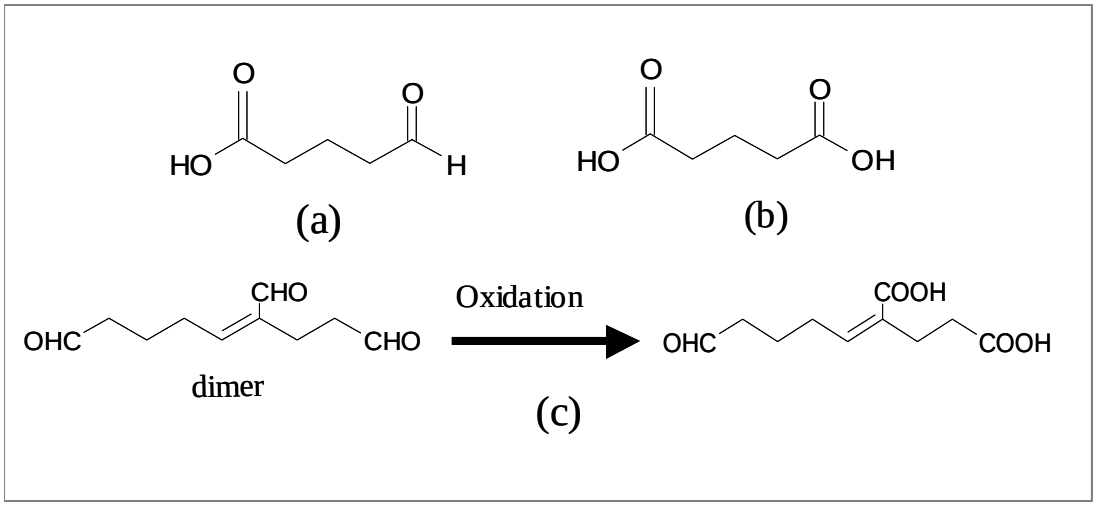


Fig. 5.86 Structure of: (a) the hemi-acid; (b) di-acid derivatives; (c) the oxidation product of higher oligomers of glutaraldehyde.

A glucose calibration curve is shown in Fig. 5.87 with a linear range from 0.1 to 0.5 mM. Application of a high voltage of 2 kV allowed glucose detection 58 s after injection.

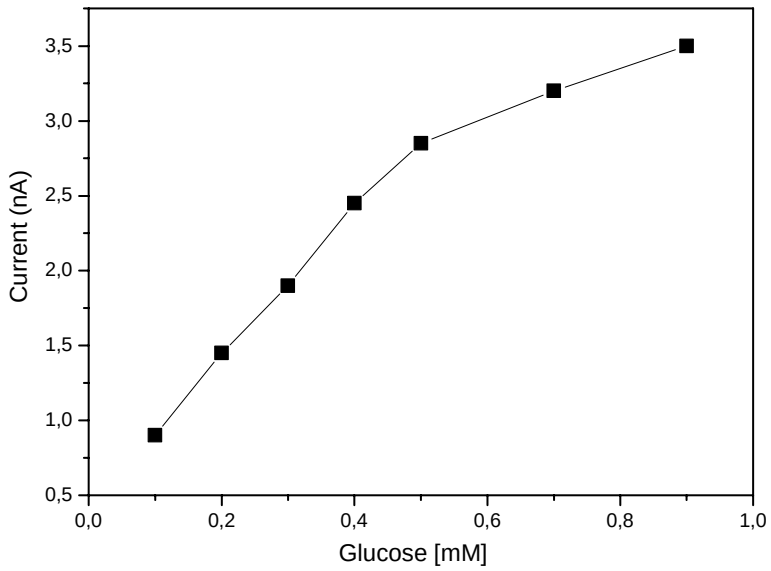


Fig. 5.87 Calibration curve obtained for glucose in a fused silica capillary. GOD was immobilized using glutaraldehyde. Phosphate buffer (10 mM, pH 7.3) was used as running buffer + 800 mV (vs. Pt-wire reference electrode) were applied to the Pt-working electrode.

5.2.4.2.2 Immobilization in glass microchips

The same method used for the immobilization enzyme by glutaraldehyde in fused silica capillary was transfered to glass microchips except the silanization step was shortened to 10 min because prolonged reaction times blocked the capillary of glass microchips. The activity

of the enzyme was investigated by the injection of glucose solutions. The signal appeared after approx. 40 s, when a voltage of + 4 kV was applied. The resulting calibration curve for glucose is shown in Fig. 5.88, where the signals increased with increasing glucose concentration with a linear rang from 0.3 to 0.7 mM with a correlation coefficient of 0.999 and standard deviation of 0.0861 were obtained. Using higher glucose concentrations (0.8 mM) the signal showed a slight decrease. Signals did not change, when the chip was stored in the running buffer at 4 ^{o}C for one day (Fig 5.88).

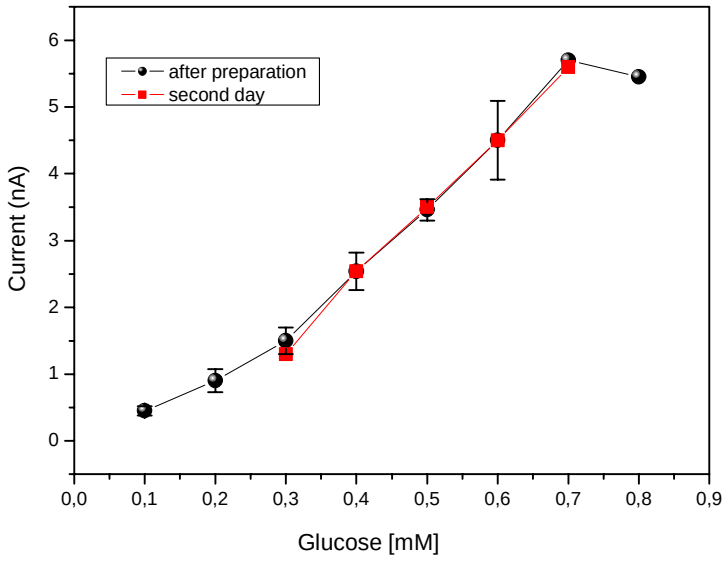


Fig. 5.88 Calibration curve obtained for glucose. GOD was immobilized in glass microchips using glutaraldehyde. 2.5 mM MES + 2.5 mM phosphate pH 7.3 was used as a running buffer, + 4 kV were applied for 30 s as injection voltage and then + 4 kV as separation voltage.

5.2.4.3 Entrapment in acrylamide gel

5.2.4.3.1 Fused silica capillary

A polyacrylamide gel was formed by the polymerization of acrylamide [as a monomer] and N, N`-methylenebismethacrylamide as a cross linker. The polymerization was initiated by the free radical formed by addition of APS and TEMED (Fig. 5.89a). Prior to the polymerization the fused silica capillary was treated by γ-methacryloxypropyltrimethoxysilane (MAPTS), which increased the adhesion of acrylamide gel to the capillary surface (Fig. 5.89b), because acrylic groups were generated on the surface and integrated in the polymer.

The polymer carried positively charged amino groups, which suppressed the EOF and thus no transportation for H_2O_2 was observed using normal EOF. To increase the EOF again the effects of some negatively charged additives were examined.

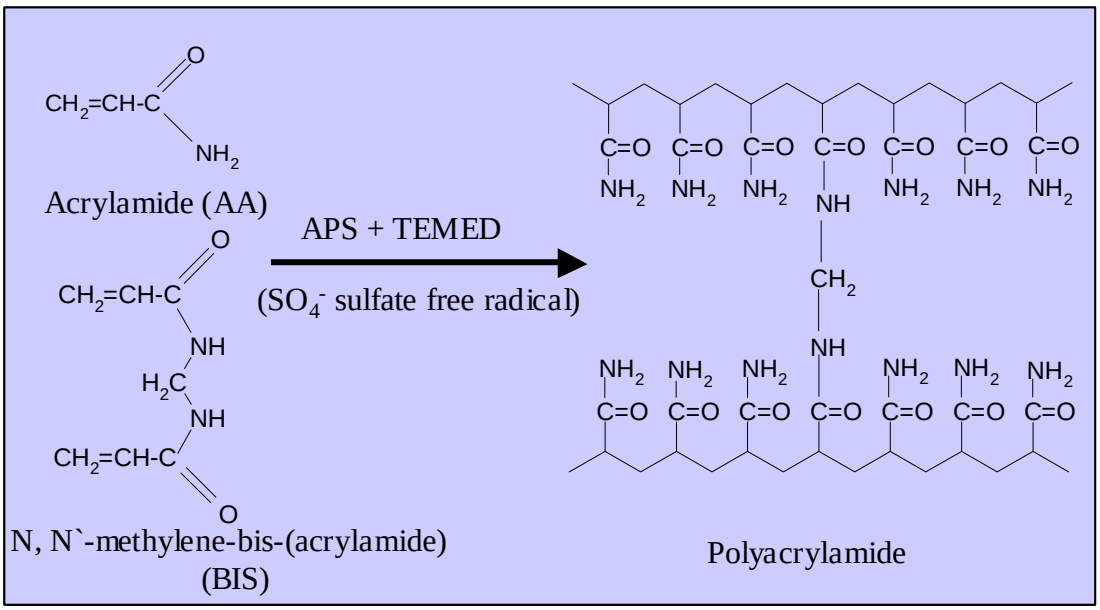


Fig. 5.89a Polymerization of AA/ BIS using free radical initiation.

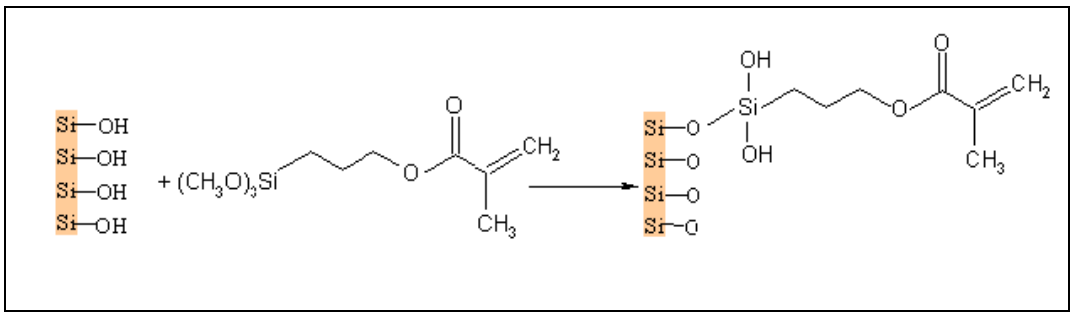


Fig. 5.89b Reaction scheme for γ-methacryloxypropyltrimethoxysilane (MAPTS) with a fused silica capillary surface.

The addition of even 10% SDS did not lead to an observable EOF as no signal was obtained even after to 30 min, whereas addition of dextran sulfate (5%) allowed the transportation of H_2O_2 within 36 s using + 800 V as a high voltage. However, the glucose signal was very small, thus, dextran sulfate introduced negative charge into the polymer, but the enzyme activity was not sufficient as already observed in chapter 5.2.4.1.3.

As glutaraldehyde in its oxidized form could also introduce negative charges (Fig. 5.85) a 2.5% solution was added to the acrylamide mixture. This allowed H_2O_2 transportation, but not as rapid as with dextran sulfate addition (2.1 min transportation time). However, the immobilized enzyme was still active, as glucose was detected also (2.2 min transportation time). After storage, these capillaries could not be used again, as bubbles formed within the

gel leading to a disruption of the electric field. This problem of bubble formation in polyacrylamide gels was studied before by many authors (Drossman et al. 1990). Bubble formation in capillary gel electrophoresis is due to volumetric losses during polymerization, which lead to shrinking of the gel during polymerization or electrophoresis. Some authors described preparation of polyacrylmide gels without bubbles. Baba et al. (1992) avoided bubbles formation by using a well-designed injection equipment, which was developed for filling capillaries by a polymerizing mixture. Preparation of polyacrylamide gradient gel-filled capillaries was described for gels below 13% T + 5% C by Chen (1998). Using allyl-β-cyclodextrin as a cross-linker instead of N,N`-methylenebisacrylamide led to gels without bubbles (Vegvari and Hjerten 2002). To prepare a polyacrylmide gel without bubbles, glycerol was added to the acrylamide mixture with GOD and glutaraldehyde before polymerization. H_2O_2 was still transported (4.2 min transportation time) and also glucose could be detected (after 4.3 min). However, the signals were very small in comparison to signals in case of glutaraldehyde as additive and bubble formation was only delayed, as it occurred after three days. Another acrylamide mixture consisting of 5% T and 60% C and 29% butylmethacrylamide in presence of 3% PEG was prepared. In this macroporous polyacrylamide gel no bubbles formed during polymerisation but no EOF was found. The non bubbles formation may be due to the presence of PEG, which is known to associate a large number of water molecules around its strands, which make the gel less hydrophobic during polymerization. It probably retains the water molecules around the growing polymer chains (Palm and Novotny 1997). To generate the EOF the influences of different negatively charged monomers were examined. These negatively charged monomers included 2-acrylamide-2-methyl-1-propanesulfonic acid) (AMPS), vinyl sulfonic acid (VSA) and acrylic acid. The chemical structures for these polymers are shown in Fig. 5.90. They were described to generate an EOF in capillary electrochromatography columns filled by polymer monoliths (Peters et al. 1997; Yu et al. 2000; Palm and Novotny 1997; Robb et al. 2002), or filled with acrylamide gels (Fujimoto 1995).

The addition of AMPS generated an EOF, as H_2O_2 was transported. But when enzyme was added to the acrylamide mixture with AMPS, no signal was detected for glucose. VSA was added to the poly(acrylamide) mixture in a concentration range from 1 to 10 wt%. Again H_2O_2 was transported with a migration time of 4 min, but no glucose signal was obtained when the enzyme was immobilized (Table 5.19).

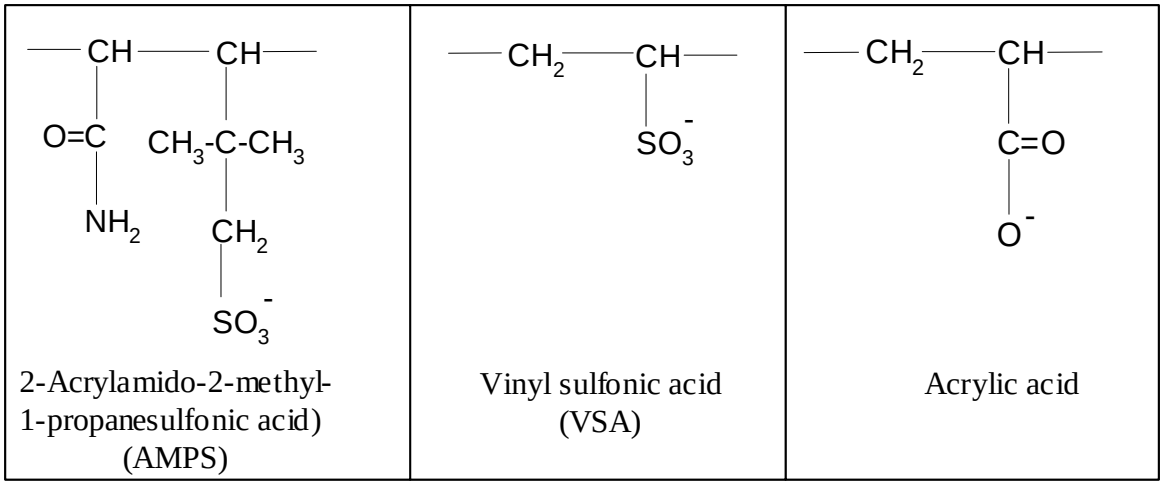


Fig. 5.90 Structure for some negative monomers.

The effect of AMPS and VSA on the activity of GOD were studied by addition of different concentrations to a phosphate buffer containing 100 mg/mL GOD and the signals for 1 mM glucose were recorded using Pt-wire working electrode. Addition of 0.025 and 0.1 wt% VSA (Fig. 5.91) had no significant effect on the activity of the enzyme, while after addition of 1 wt% no activity was observed. To generate an EOF using VSA concentrations higher than 1% were used in a continuous bed for capillary electrochromatography (Kornysova et al 2001; Zhang and El Rassi 2002). For AMPS, the addition of 0.025, 0.1 and 1 wt% reduced the activity by ~ 31, 46 and 96%, respectively. To generate an EOF an amount of 1 wt% from AMPS was used (Bedair and El Rassi 2002). Obviously both compounds reduced GOD activity when used in concentrations required for EOF generation.

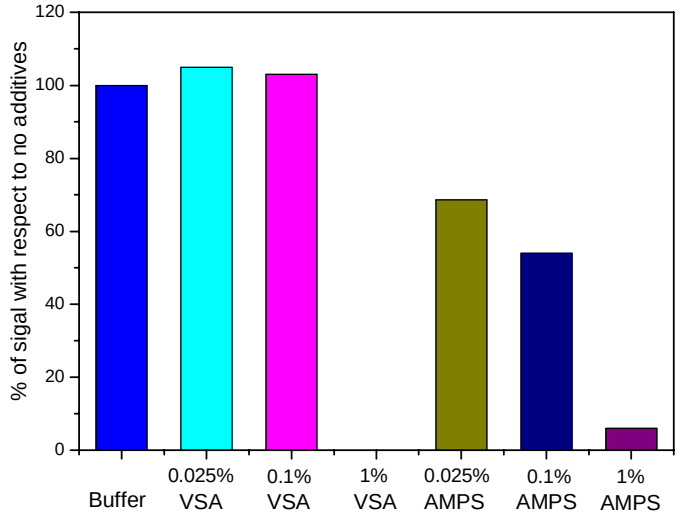


Fig. 5.91 The influence of some negative monomers on the activity of GOD 100 mg/mL using 1 mM glucose, Pt-working electrode with + 700 mV.

Poly(acrylic acid) was also described to generate an EOF in a capillary electrochromatography column filled by a macroporous polyacrylamide/PEG stationary phase (Palm and Novotny 1997). An acrylamide mixture of 5% T, 60% C, 3.9% acrylic acid, 3% PEG and GOD 100 mg/mL was polymerized in the presence of 10% APS and 10% TEMED (chapter 4.3.6.8). The formed macroporous gel showed no bubble formation during preparation, storage or during electrophoresis. Using this capillary, a glucose signal was obtained after 58 s. Poly(acrylic acid) is the main source for the EOF and the presence of PEG increased the porous structure which increased the EOF (Bedair and El Rassi 2002). In a mixture of glucose and ascorbic acid two separate signals were obtained (Fig. 5.92).

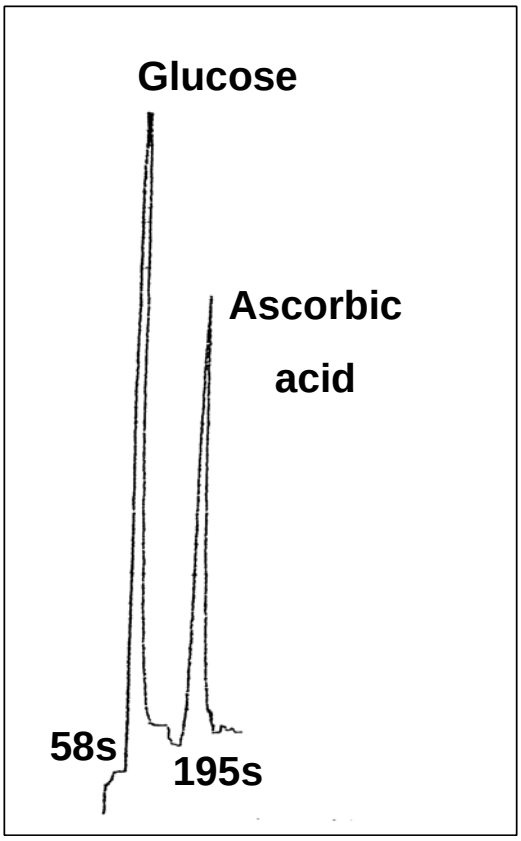


Fig. 5.92 Separation of 0.5 mM glucose and 0.5 mM ascorbic acid in a fused silica capillary with GOD immobilized in an acrylamide gel with poly(acrylic acid). The injection voltage of + 800 V was applied for 20 s, which then used as a transportation HV.

A glucose calibration curve showed a linear range form 0.2 mM to 2.0 mM (Fig. 5.93) with a correlation coefficient of 0.997 and a standard deviation of 0.56.

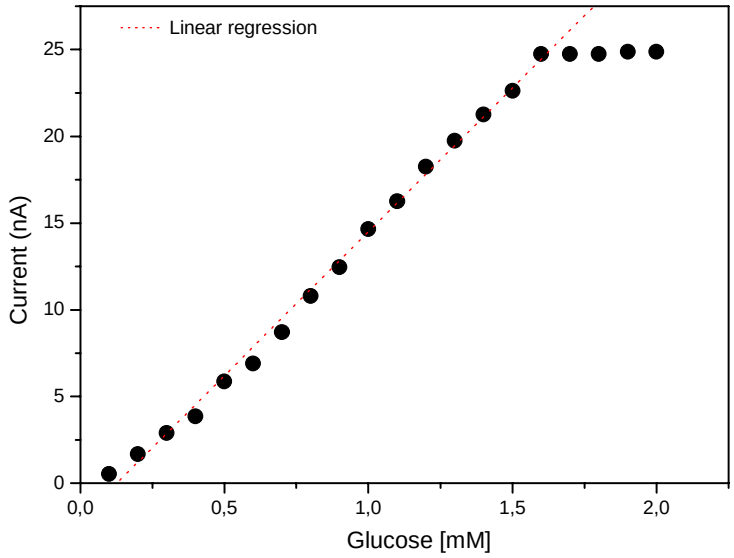


Fig. 5.93 Calibration curve obtained for glucose with GOD immobilized in an acrylamide gel with poly(acrylic acid) in a fused silica capillary.

5.2.4.3.2 Glass microchips

The same acrylamide mixture used for the immobilization of GOD in fused silica capillaries was applied to glass microchips. Channels were pre-treated by MAPTS before the polymer mixture was introduced into the channel and allowed to polymerize overnight in the used buffer. The part in the microchips where the enzyme was immobilized is shown in Fig. 5.94. The sample was injected by application + 800 V for 20 s on the SR while BR and SW were floated and BW was grounded. + 2 kV separation voltage was applied on the BR while BW was grounded and 85% of the used separation voltage was applied on both SR and SW.

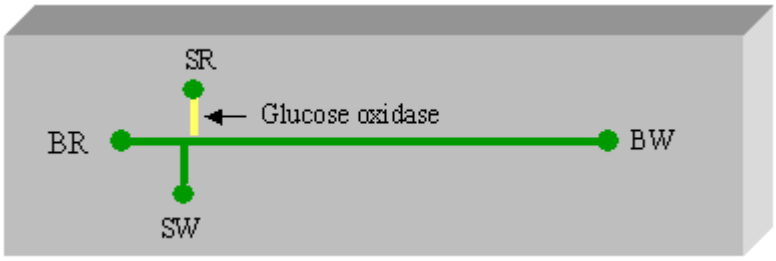


Fig. 5.94 The part in glass microchips where GOD was immobilized in a polyacrylamide gel.

This allowed glucose detection after 76 s, while ascorbic acid was detected after 300 s. The calibration curve (from 0.05 mM to 2.0 mM) showed linear range from 0.1 mM to 1.1 mM with a correlation coefficient of 0.998 and 0.9163 as standard deviation (Fig. 5.95).

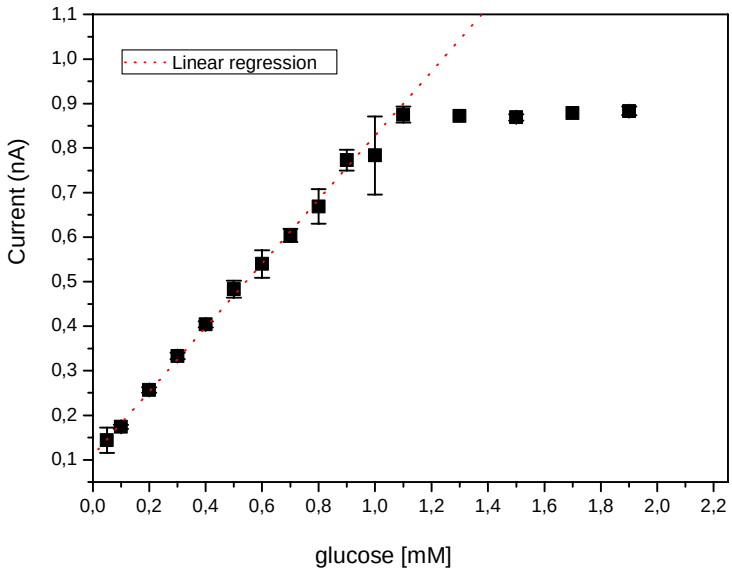


Fig. 5.95 Calibration curve obtained for glucose with GOD immobilized in an acrylamide gel in glass microchip. The injection voltage of + 800 V was applied for 20 s and + 2 kV was used as a separation voltage.

Glucose was determined in 3 different juice samples (No. 5, 6 and 7 in Table 5.1) using this chip. The obtained results were compared with the result obtained from YSI. The obtained data showed good agreements between the two systems (Fig. 5. 96).

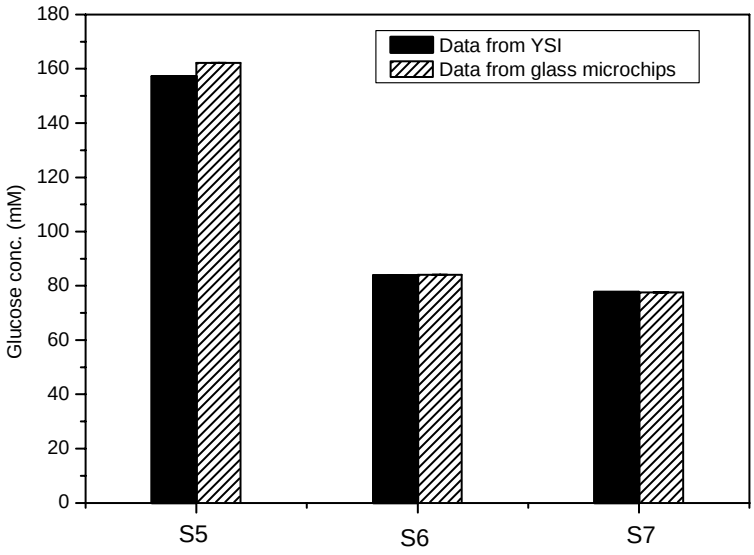


Fig. 5.96 Determination of glucose in three different juice samples using YSI and glass microchips immobilized in an acrylamide gel.

The stability of the immobilized enzyme was tested by three different ways. The short-term stability of the enzyme was examined by continuous work for 8 h and no loss of activity was

observed (Fig. 5.97). Moreover a good reproducibility for the migration times for glucose and thus a stable EOF was found. Additionally, the stability of the enzyme chip was examined daily for 3 weeks with the chip being stored in phosphate buffer at + 4 ^{o}C. After 20 days the enzyme had still ~ 77% from its initial activity (Fig. 5.98). The long-term stability of the enzyme was examined further during 7 weeks of storage under the same previous conditions. The immobilized enzyme showed no further loss of its activity and had ~ 80% of its initial activity after 7 weeks (Fig. 5.99).

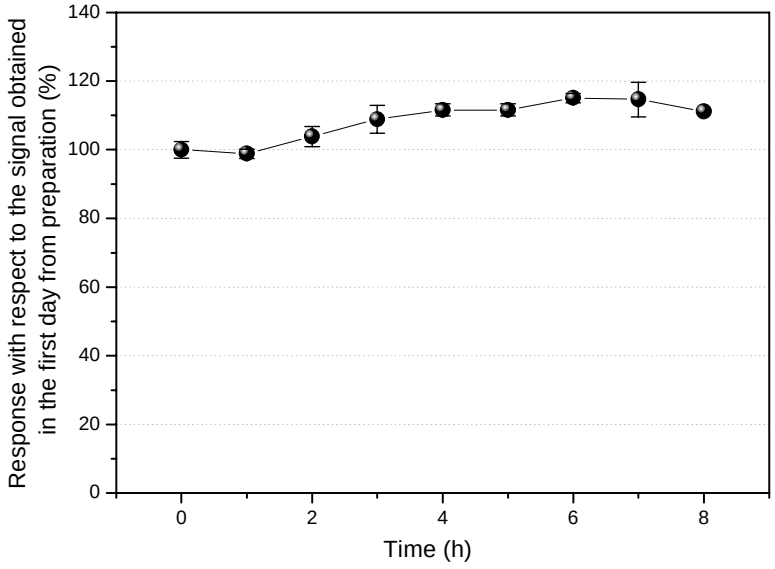


Fig. 5.97 Stability of GOD immobilized glass microchips after 8 h continuous work.

5.2.4.3.3 Summary

Immobilization of enzyme in fused silica capillaries and glass microchips was achieved using glutaraldehyde. Also the immobilization of enzyme using a polymer dispersion based on polyurethane and an ink based on diacrylate, which had been previously used for enzyme electrodes was possible. Acrylamide gel was another material used for the immobilization of GOD in fused silica capillary and glass chips. The effects of different negatively charged additives such as dextran sulfate, glutaraldehyde, AMPS, VSA and acrylic acid were examined to generate the EOF in the gel structure (Table 5.19). The best additive was acrylic acid as it generated the EOF and maintained enzyme activity. The immobilization method was applied in glass microchips and glucose was detected in 3 juice samples and showed a good correlation with the standard method (YSI).

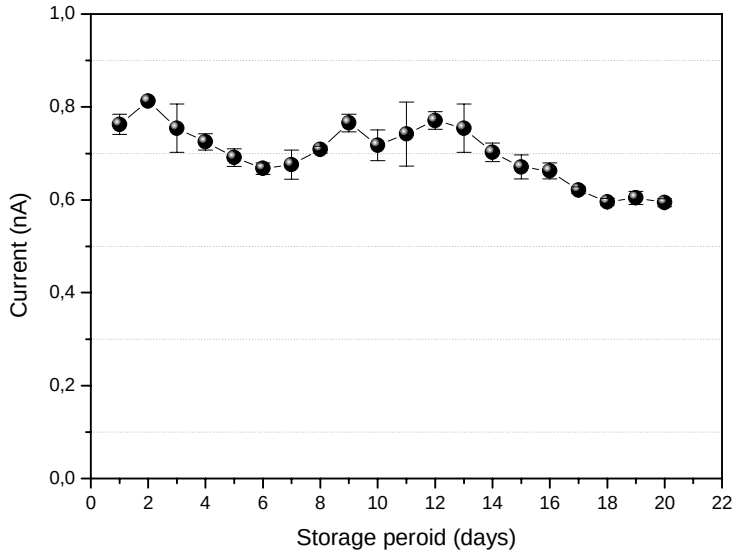


Fig. 5.98 Stability of GOD immobilized in glass microchips in an acrylamide gel after 22 days of storage in phosphate buffer at 4 ^{o}C.

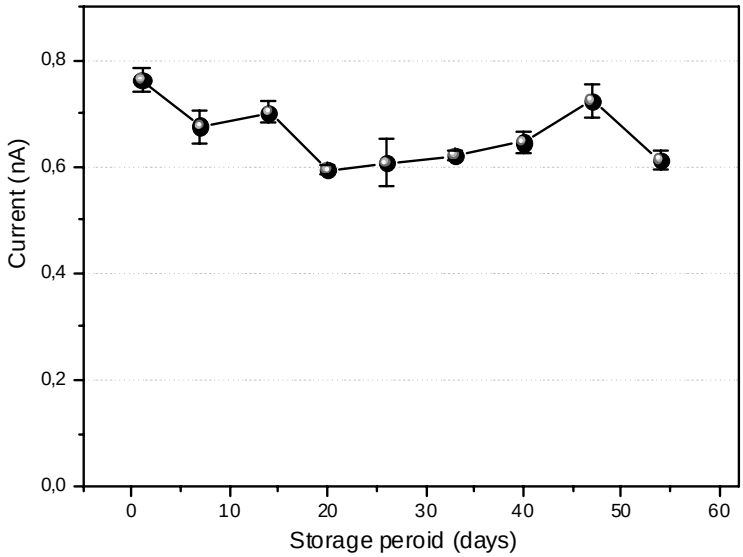


Fig. 5.99 Long-term stability for GOD immobilized in glass microchips in an acrylamide gel after 7 weeks of storage in phosphate buffer pH 7.3 at + 4 ^{o}C.

Polymer composition	Capillary treatments	HV used (V)	Migration time for H_2O_2	Migration time for glucose	EOF and enzyme activity
AA/BIS + SDS (10%) GOD 100mg/mL	without	+ 800	No signal	No signal	No EOF
AA/BIS + Dextran sulfate 5%	without	+ 800	36 s	7.30 min small signal	EOF, low enzyme activity
AA/BIS + GA 2.5% + GOD 100 mg/mL	without	+ 800	2.10 min	2.25 min	EOF and enzyme activity, bubbles formed
AA/BIS + Glycerol 10% +GA 2.5% + GOD 100 mg/mL	Silanization	+ 1000	4.18 min small signal	4.3 min small signal	EOF and low enzyme activity, bubble formed after 3 days
AA/BIS + AMPS + GOD 100mg/mL	Silanization	+ 600	2 min	No signal	EOF but no enzyme activity
AA/BIS + AMPS + GA+ GOD 100 mg/mL	without	+ 600	1 min	No signal	EOF but no enzyme activity
AA/BIS + (1-10%) VSA + GOD 100 mg/mL	Silanization	+ 500	4 min	No signal	EOF and no enzyme activity
AA/BIS (5% T and 60% C) + VSA + Butylmethacrylamide +PEG + GOD 100 mg/mL	Silanization	+ 800	11 min	No signal	EOF and no enzyme activity
AA/BIS (T 5%, C 60%) + PEG + GOD 100 mg/ mL+	Silanization	+ 800	No signal	No signal	No EOF
AA/BIS (5% T and 60% C) + PAA + PEG + GOD 100 mg/mL	Silanization	+ 800		58 s	EOF and enzyme activity

Table 5.19 The polymerisation composition, the resulted EOF and enzyme activity using polyacrylamide gel.

5.2.5 Separation of carbohydrates using direct electrochemistry in fused capillary and glass microchips

Capillary electrophoresis was used for the separation of carbohydrates with electrochemical detection using a copper electrode (Colon et al. 1993; Huang et al. 1995; Fu et al. 1998; Voegel et al. 1997) and strong alkaline solutions (pH ca.13).

With the CE set up with fused silica capillaries shown in Fig. 3.3 several trials were done to separate and detect carbohydrates. With 10 mM NaOH as running buffer glucose, fructose and sucrose were transported by EOF to the electrode but the separation was not possible due to the closed migration times for these carbohydrates. Using NaOH 100 mM the separation was also not complete. By addition of 5 mM MES to 100 mM NaOH glucose was separated from sucrose (Table 5.20a). By 50 mM NaOH and 0.2 mM CTAB a reversed EOF was obtained and glucose, fructose and sucrose were transported and completely separated using fused silica capillary electrophoresis.

In another experiment ascorbic acid glucose and sucrose were separated using NaOH 50 mM in the presence of 0.5 mM CTAB using -100 and - 200 V separation voltages (Fig. 4.100).

Using sodium tetraborate (pH 8-10) which forms complexes with carbohydrates, no amperometric signal with a copper electrode was obtained. This may be due to the change in the electrochemical behavior of the produced complexes. Thus, the electrochemistry of carbohydrates was investigated in a buffer prepared from 100 mM sodium tetraborate (pH 10) and another borate buffer prepared by dissolving boric acid (100 mM) and adjusting the pH to 10 by NaOH. The effect of the electrode potential applied to a Cu-electrode on the signal for 10 mM glucose was examined in the range from 0.0 V to + 0.8 V. With tetraborate solution no signals were obtained in the examined electrode potential range. Using the boric acid solution glucose signals increased by increasing the electrode potential. Thus, the electrochemistry for glucose was different in both borate solutions. In sodium tetraborate a reduction of glucose sensitivity was obtained. The borate-carbohydrate complex depends on the salt used to make up the aqueous solution and on the pH (Paulus and Klockow-Beck 1999). At high pH-values the formation of tetrahydroxy borate ions is favoured (chapter 2.2.2.3.6). Boron can form a multitude of stable polynuclear complexes with oxygen, such as tetraborate. Tetraborate forms only above pH 12 tetrahydroxy borate ions. This may explain why no signals were obtained for carbohydrates using capillary electrophoresis with sodium tetraborate buffer. Borate buffer prepared from boric acid (100 mM) and NaOH was used as running buffer in capillary electrophoresis for separation of carbohydrates using + 800 mV electrode potential for amperometric carbohydrate detection. The migration time for glucose,

fructose and sucrose were 150 s, 158 and 75 s, respectively, thus, no separation was obtained between glucose and fructose.

Using a capillary filled with a polyacrylamide gel (AA/BIS with polyacrylic acid to generate an EOF) the three sugars were transported but signals were not completely separated. Addition of metal ions such as Ca^{++} or Ba^{++} did not increase the resolution but reduced the EOF as positive charges on the inner capillary surface were increased.

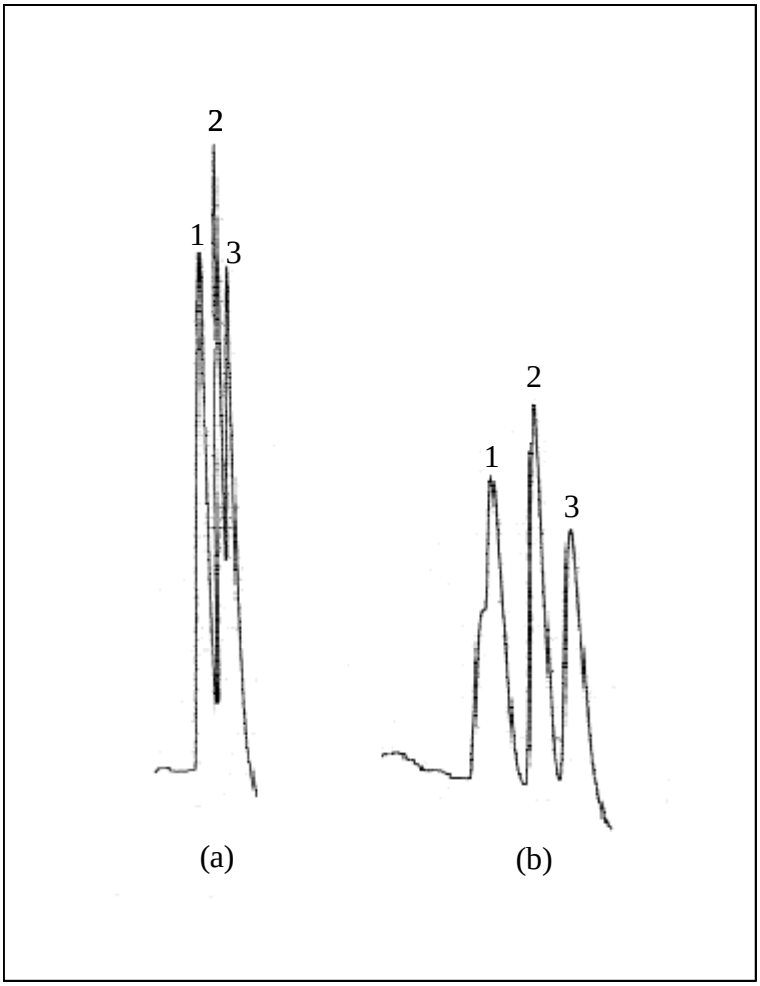


Fig. 5.100 Separation of ascorbic acid (1), glucose (2) and sucrose (3) using fused silica capillary with 50 mM NAOH + 0.5 mM CTAB. Separation high voltage were – 200 V (a) and – 100V (b). Migration times as mentioned in Table 5.20a.

Buffer used	HV (V)	Carbohydrates	Results
NaOH 10 mM	+ 600	Fructose Glucose Sucrose	135 s 133 s 149 s
NaOH 50 mM	+ 600 + 400	Glucose Sucrose Glucose Sucrose	324 s 362 s 445 s 483 s
NaOH 50 mM + 0.2 mM CTAB	- 100	Fructose Glucose Sucrose	443 s 544 s 847 s Separated
NaOH 50 mM + 0.5 mM CTAB	- 100	Glucose Sucrose Ascorbic acid	165 s 255 s 300 s Not completely separated (Fig. 4.100a)
NaOH 50 mM + 0.2 mM CTAB	- 200	Glucose Sucrose Ascorbic acid	549 s 675 s 345 s Completely separated (Fig. 4.100b
NaOH 100 mM + 5 mM MES	+ 300	Glucose Sucrose	228 s 185 s Not completely separated
NaOH 100 mM + 5 mM MES	+ 200	Glucose Sucrose	428 s 340 s Completely separated
Sodium tetraborate pH 8-10	+ 600	Glucose Sucrose	No signal
Borate buffer (Boric acid) pH 10	+ 800	Glucose Fructose	150 s 132 s No complete separation
Borate buffer (Boric acid) pH 12	+ 800	Glucose Fructose Sucrose	150 s 158 s 75 s No separation between glucose and fructose
Polyacrylamide gel AA/BIS + PAA (NaOH 50 mM)	+ 500	Glucose Fructose Sucrose	160 s 160 s 120 s
Polyacrylamide gel AA/BIS + PAA + Ba^{+} or Ca^{+} (NaOH 50 mM)	+ 500	Glucose Fructose Sucrose	219 s 189 s 169 s
NaOH 100 mM	+ 400	Glucose Fructose Sucrose	240 s 220 s 210 s

Table 5.20a Separation of carbohydrates using fused silica capillary electrophoresis.

Application of the same conditions as used for the separation of carbohydrates in fused silica capillaries to glass microchips led to no separation. With NaOH (100 mM) + MES (5 mM) the migration times for the studied carbohydrates were very close to each others (from 70 to 79 s). The same result was obtained with NaOH (20 mM) + sodium acetate (10 mM). Using 20 mM NaOH + 0.01 mM CTAB the difference in migration times increased but no real separation was obtained. At last by using NaOH 50 mM + (0.2 or 0.5) mM CTAB and with negative high voltage (reversal EOF) a broad peak was obtained and no complete separation was obtained (Table 5.20b).

The reason for this difference in the behavior in fused silica capillaries and glass microchips is the difference in the chemical compositions of glass microchips and fused silica capillaries. This difference in the chemical compositions led for example to a blockage of the glass microchips by silanization using the same method as for fused silica capillaries. This chemical composition difference also affects the behavior of carbohydrates when the same conditions were used in fused silica capillaries and in glass microchips.

Buffer used	HV (V)	Carbohydrates Examined	Results (migration time)
NaOH 20 mM + 5mM MES	+ 2 kV	Glucose Fructose Sucrose	79 s. 70 s. 67 s. Not separated
NaOH 20 mM + 10mM NaAc	+ 2 kV	Glucose Fructose Sucrose	77 s. 76 s. 73 s. Not separated
NaOH 20 mM + 10 mM NaAc + 0.01 mM CTAB	+ 2 kV	Glucose Fructose Sucrose AA	126 s. 117 s. 103 s. 122 s Not separated
NaOH 50 mM + 0.2- 0.5 mM CTAB	- 1 kV	Glucose Ascorbic acid	315 s 310 s Not separated
NaOH 50 mM + 0.2- 0.5 mM CTAB	- 2 kV	Glucose Ascorbic acid	86 s 42 s Not separated

Table 5.20b Separation of carbohydrates using glass microchips.

5.2.6 Summary and conclusion

Determination of glucose by enzymatic reactions using capillary electrophoresis with amperometric detection using fused silica capillaries and glass and PMMA microchips was

studied. Firstly glucose was detected using an enzyme solution as running buffer. Presence of GOD in the running buffer led to a gradual increase in the migration time for the enzymatically produced H_2O_2, thus, the EOF decreased with repeating the experiment. This decrease in the EOF was due to the adsorption of enzyme onto the inner surface with time. Using the cationic or anionic surfactants, adsorption was eliminated and a stable EOF resulted. Addition of low CTAB concentrations to the running buffer and normal EOF prevented enzyme adsorption on the inner capillary surface of glass and PMMA microchips. The addition of CTAB also enhanced the separation efficiency between librated H_2O_2 and ascorbic acid. The migration time for ascorbic acid was much higher in the presence of CTAB than in the absence of it. The effect on the migration time for H_2O_2 was lower. The interaction between the negatively charged ascorbic acid and the positive charge on the surface from CTAB may increase the migration time for ascorbic acid, while in the case of H_2O_2 this additional interaction did not take place. Glucose was successfully detected using glass and PMMA microchips in three different juice samples. The obtained results showed a good correlation with the data from the standard method (YSI).

Immobilization of enzyme in the injection channel was another method, which was examined for the elimination of enzyme adsorption onto the inner capillary surface. Different protocols using different materials were investigated. Immobilization of GOD by cross-linking with glutaraldehyde was achieved. Immobilization of GOD by entrapment in a UV paste or polymer was also studied. The entrapment of GOD in a poly(acrylamide) gel gave the best results in the presence of an EOF generator. Negative additives such as AMPS and VSA generated the EOF, but a loss of enzyme activity was observed. Only presence of poly(acrylic acid) generated an EOF and maintained the enzyme activity. GOD was immobilized through the sample injection channel in glass microchips. The immobilized enzyme showed a high stability up to ~ 2 months when the chips were stored in the buffer at 4 ^{o}C. Glucose was detected in juice samples using glass microchips in which GOD was immobilized in the presence of poly(arylic acid) and PEG. Highly reproducible results were obtained and showed a good correlation to YSI.

Separation of carbohydrates by amperometric detection after direct transportation using high alkaline solutions for charging carbohydrates or complexation with borate ion was also investigated. Using fused silica capillaries some carbohydrates were separated but in glass microchips it was not possible as shown in Tables 5.20a and b.

6 SUMMARY

This work contains two main parts: determination of glucose using an enzyme electrode and determination of glucose in microchips capillary electrophoresis.

For the enzyme electrodes screen-printed Pt-electrodes were used as transducers on to which GOD was immobilized by entrapment in a paste based on polymethacrylate and polymerized by UV-irradiation. Immobilization of enzyme only led to high permeabilities for other electroactive compounds which are oxidized at the same potential used for H_2O_2 determination such as ascorbic acid and xanthine. The effects of some negative, neutral and positive additives were investigated. The addition of 10% Gafquat 755N resulted in a higher sensitivity for glucose (~ 4-fold increase) but a lower selectivity was obtained. Addition of a 1% solution of the positively charged poly-L-lysine to the enzyme membrane showed a 6.5-fold increase in glucose signals. Also when BSA or SDS were added, an enhancement of the sensitivity and a loss of selectivity was found. Three different additives were added to the enzyme membrane and showed a good sensitivity, selectivity and stability. These additives include the neutral polymer PEG, the negatively charged polymer Nafion and graphite powder. Addition of 10% PEG showed a 3-fold increase in the sensitivity of glucose signals. Presence of 0.1 g/ L ascorbic acid increased glucose signals by ~ 0.15 to 3.75% from the signals in the absence ascorbic acid. By addition of 10% PEG to the enzyme membrane the remaining activities were 90% and 62% after 8 and 90 days, from storage, respectively. Addition of 5% Nafion to the GOD membrane showed a lower sensitivity but the enzyme electrodes showed a good stability after 90 days of storage. Presence of ascorbic acid 0.1 g/L increased glucose signals by ~ 0.05 to 5% from the signals in the absence of ascorbic acid. Graphite powder 0.5% was the best additives as the highest sensitivity (~ 7-fold the signals without graphite) and a complete elimination of effects of the presence of ascorbic acid on the glucose response obtained. The enzyme electrode delivered ~ 90% of their original signals after 90 days of storage. Using the optimal conditions for glucose electrode in the presence of poly-L-lysine + BSA, PEG, Nafion and graphite, glucose was detected in 7 juice samples and in cultivation samples from *E. coli*. The first electrode showed poor results in comparison to the data from YSI due to the lower selectivity of the enzyme electrode while the other three electrodes showed good correlations to the data obtained from YSI.

As an alternative the determination of carbohydrates (especially glucose) using capillary electrophoresis using fused silica capillaries or different types on microchips was investigated. Determination of glucose using an enzyme solution was investigated in glass and PMMA microchips. To reduce enzyme adsorption onto the inner capillary surfaces due to the

interaction between the negative silanol groups and amino groups from the enzyme in case of fused silica capillaries or glass microchips and the hydrophobic interaction between the enzyme and the surface of PMMA microchips, the influences of cationic and anionic surfactants were examined. The addition of the cationic single-chained surfactant CTAB showed a reduction the EOF at low CTAB concentrations and a reversal of the at higher CTAB concentrations. The effect of the double-chained surfactant DDAB was also examined using glass microchips. At low DDAB concentrations no significant effect on the EOF was observed, but at higher concentrations the EOF was reversed. The negatively charged surfactant SDS led to an enhancement of the EOF till the capillary surface was saturated.

Coating of the capillary surface by either CTAB or SDS also affected the separation of signals originating from H_2O_2 or ascorbic acid oxidation. The introduction of positive (CTAB) or negative (SDS) charges slowed down or accelerated the transportation of ascorbic acid and H_2O_2 to different degrees, probably due to additional electrostatic interactions between ascorbic acid and the capillary wall. Thus, the addition of CTAB increased the difference in migration times, whereas a decrease was observed after addition of SDS.

Thus, glucose could be detected in different juice samples when low amounts of CTAB were added to the running buffer together with GOD. Data showed a good correlation with the data from YSI used as reference.

To avoid continuous addition of GOD to the running buffer, different approaches were used for the immobilization of GOD in the capillaries. Prior to enzyme immobilization capillary surface were silanized to obtain amino functions or acrylic functionalities on the inner surfaces. Best results were obtained by covalent attachment and cross-linking of the enzyme using glutaraldehyde and by entrapment of the enzyme in an acrylamide gel. As the analyte was to be transported by electrokinetics, negative charges had to be introduced into the immobilization matrix, so that an EOF was established.

Additionally, the enzyme activity had to be maintained. Stable acrylamide gels with sufficiently active GOD resulted from the addition of poly(acrylic acid), while other additives either inactivated the enzyme or led to instable gel structures. Thus, microchips with GOD immobilized in the sample-introduction channel in an acrylamide gel supplemented with poly(acrylic acid) were applied to glucose determination in fruit juice and data correlated well to those obtained with the reference method.

Electrophoretic separation of some carbohydrates was also investigated using direct electrochemical detection of carbohydrates after charging by using pH higher than 11 or complexation with borate ions. Using fused silica capillaries and NaOH 50 mM + 0.2 mM

CTAB, glucose, fructose and sucrose were separated and detected using amperometric techniques with a copper wire as a working electrode. Also separation between glucose and sucrose was accomplished using NaOH 50 mM in of 5 mM MES buffer. Sucrose was separated either from glucose or fructose using borate pH 12 but no complete separation of all three compounds was obtained.

The presence of electrochemical interferents in real samples is a common problem of enzyme electrodes. In the present work it was solved either by modification of the composition of the immobilization matrix on top of a screen-printed electrode or by electrophoretic separation of the interfering compound from the analyte in microchips. The enzyme electrodes were integrated in an automated FIA-device and are thus applicable to the automated analysis of samples, as required for example for on-line bioprocess monitoring. For microchips suitable interfaces allowing automated analysis are still missing, however, short analysis times are obtained and low sample volumes are required as important in medical diagnosis, drug screening or on-site analysis of samples.

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